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13. ABSTRACT *(Maximum 200 words)*

Heat shock proteins (hsps) are induced in cells in response to environmental stresses. It has been shown that some breast cancer patients express high levels of hsp27, which may augment the aggressiveness of these tumors and make them more resistant to treatment. The research funded by this fellowship was directed at understanding the regulation of hsp27 toward the development of a useful therapy for inhibiting breast cancer progression. In this our final progress report we demonstrated completion of Task 1--the examination of the regulatory mechanisms controlling the expression of hsp27 in breast cancer cells. On Task 2 we examined genes whose expression is associated with hsp27 effects on cellular proliferation, metastatic behavior, and drug resistance. We applied the technique of screening expression microarrays to identify candidate cDNA's which were modulated as a result of hsp27 overexpression and/or drug treatment. Furthermore, we have determined that hsp27's effect on drug resistance involved the inhibition of drug-induced apoptosis. Finally, we completed Task 3, cloning of a hsp27 regulatory factor which interferes with hsp27 expression.

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FOREWORD

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Suzanne Trigueiro 10/19/98

PI - Signature Date

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INTRODUCTION:

Heat shock proteins (hsps) are induced by a variety of environmental and physiological stresses (1), and the small molecular weight hsp27 is often found at high levels in human breast tumors (2) conferring a poor prognosis to certain breast cancer patients. We have demonstrated that hsp27 is involved in regulating the growth of breast cancer cells as well as the development of drug resistance in these cells (3). Recently, we have also discovered a role for hsp27 in the invasion and metastatic behavior of breast cancer cells (4). Based on these findings, we hypothesized that hsp27 plays a pivotal role in breast cancer progression, and have focused upon its regulation, interfering with its expression, and understanding the cellular pathways through which it exerts its effects on breast cancer cells.

In this fellowship we originally proposed the following Specific Aims:

Task 1. To examine the regulatory mechanisms underlying the expression of hsp27 in breast cancer cell lines and human breast tumors. (Years 1-3).

Task 2. To identify genes whose expression is associated with hsp27 effects on proliferation and drug resistance.. (Year 3).

Task 3. To target positive and negative hsp27 transcriptional regulatory factors to interfere with hsp27 expression. (Years 2-3).

This fellowship was originally awarded to fund the postdoctoral research efforts of Dr. Carl Castles. Dr. Castles worked on Tasks 1 and 2 until he left my research group for an academic position at the Department of Biological and Physical Sciences, Montana State University-Billings, MT during the second year of this fellowship. Dr. Rhonda Hansen then joined my group, and after permission from the USAMRMC, she was allowed to continue the work on Tasks 2 and 3. Both Drs. Castles and Hansen were very productive on this project with many resulting abstracts and publications as listed in the Bibliography section.

BODY:

Task 1-Hsp27 Regulatory Mechanisms:

The first year of the grant was spent examining the regulatory elements of the hsp27 promoter. Two breast cancer cell lines, MCF-7 and MDA-MB-231 with differing endogenous hsp27 levels, were used as a model to study the molecular mechanisms important for basal promoter transcriptional activity. A genomic clone containing 1.1 kb of the hsp27 promoter was sequenced and the regulatory elements were characterized. The first 200 bp within this 5'-flanking region was found to hold the majority of the transcriptional activity, according to transient transfection assays using a series of hsp27 promoter deletion fragments in luciferase reporter vectors. This region of the hsp27 promoter contains several distinct features, including characteristics of housekeeping genes (such as SP1 binding sites), of regulated genes (TATA sequences), of heat-inducible genes (the heat shock element or HSE), and of estrogen-inducible genes (an imperfect estrogen response element or ERE). Although hsp27 is estrogen-regulated, ER does not appear to directly bind to the hsp27 promoter and stimulate hsp27 gene transcription. We have determined that the majority of the hsp27 promoter activity is contained within the most proximal 200 bp of this 5' region, and a G/C-rich region containing overlapping SP1 and AP2 sites was found to be responsible for the basal transcriptional activity. Task 1 was thus completed in Year 1 of the granting period, and the work was published (5). Detailed Experimental Methods and Figures are not included here in the Body of the Report, but are shown in (5) included in the Appendix.

Because we found that the majority of hsp27 transcriptional activity was the result of regulation through this most proximal 200bp region, we next focused our attention to this region which lead to the cloning of a new hsp27 transcriptional regulator protein during Years 2 and 3, as will be described in detail in Task 3.

Task 2-Identify Genes Associated with Hsp27 Effects on Proliferation and Drug Resistance

Summary:

Task 2 was originally planned to begin in Year 3, however with our rapid success completing Task 1, we simultaneously began work on Tasks 2 and 3 in Year 2. We decided to utilize a bipartite approach to accomplish our objectives in Task 2. In our first approach, we utilized differential display (DD) to detect genes whose levels varied in response to hsp27 expression in stable transfectants overexpressing hsp27 cDNA. This lead to two publications (6, 7) included in the Appendix. Our efforts using differential display analyses were then replaced with a more sensitive and inclusive technique, screening of cDNA microarrays. This work has so far resulted in one abstract (8), and one manuscript in press (9).

Our second approach to Task 2 had originally been designed to use two-hybrid analyses, but we decided that this approach incorrectly assumed that hsp27 directly interacted with key pathway regulators of drug resistance and metastasis and would not identify indirect effects. So we decided to directly examine genes known to be involved in the regulation of those pathways influenced by hsp27 expression. This work has so far resulted in three abstracts (10-12), and one submitted manuscript (13).

Approach 1

To begin it was necessary to optimize the technique of DD, and the isolation of RNA for use in DD. Dr. Castles first developed a method for recovering high-quality RNA from specific cells microdissected from air-dried frozen histological sections of unfixed tissue for its use in DD (6). He then developed a method of histological staining with nuclear fast red, so as to obtain RNA from paraffin-embedded samples useful for DD (7). The detailed Experimental Methods and Results of this developmental phase of the project are shown in these two manuscripts (6, 7) and are included in the Appendix for review.

Now with the methodical hurdles behind us, Dr. Hansen next generated MDA-MB-231 cells which normally express low levels of hsp27, stably overexpressing hsp27

under the control of a constitutive cytomegalovirus promoter. The range of hsp27 overexpression varied from 2 to 5-fold in the hsp27 transfectants as compared to the control-transfected cells when immunoblots containing extracts from the transfectants were evaluated by densitometric scanning. Dr. Hansen then applied DD using a combination of twenty-four 5' arbitrary primers and three 3' anchored primers. From this initial series she identified 11 cDNA's which were differentially expressed in the hsp27-overexpressing transfectants. We focused on two different patterns of expression, those which were induced by hsp27 overexpression, and three cDNA's whose expression was suppressed in the hsp27-overexpressing transfectants. We sequenced these 11 partial cDNA's and found that they did not match any known cDNA contained within GENEBank, but showed varying similarities to expressed sequenced tags in the Bank. Thus, this sequence information was of little assistance in the prioritization of which clones to evaluate. Before beginning the arduous task of determining what role these cDNA's play in hsp27's known effect on breast cancer progression, we next tried to confirm their relative expression in our hsp27 stable transfectants and controls using Northern Blot analyses. Unfortunately, we were unable to either detect expression of these putative cDNAs using 10-20 μg of polyA+ RNA, or we did not confirm their expected differential expression in the hsp27-overexpressing transfectants. Thus, we were forced to abandon the use of DD and started analyses with expression microarrays.

cDNA expression microarrays offer the potential to simultaneously quantify expression of many genes. Advances in cDNA array technology to address issues such as array size, probe density, probe content, and readout now make this technology sufficiently flexible, accessible, and practical for application in the laboratory. Thus far, cDNA arrays of one type or another have been successfully utilized in paired comparisons analogous to our objective to identify genes associated with hsp27 expression. But although the microarray technology holds great promise for quantitative profiling of gene expression, its novelty means that there are no well-established and

widely accepted standards to guide analysis and interpretation of the data that it produces. We have thus sed preliminary data generated with the CLONTECH Atlas™ human cDNA expression array to develop a practical approach to statistical analysis of our data, and have developed the use of Principal Components Analysis (PCA) to identify genes with altered expression. This work has resulted in one publication (9) in press.

In more detail, we sought to identify those genes which were associated with hsp27's effect on drug resistance and progression. To do this, Dr. Hansen isolated RNAs from control-transfected cells and the hsp27-overexpressing transfectants. The cells were either treated with doxorubicin for 48 hours or left untreated. We chose this treatment condition because we had previously determined that hsp27 overexpression conferred resistance to doxorubicin (3). The RNAs were then used to synthesize ^{32}P-radiolabeled cDNAs for hybridization to the Atlas™ human cDNA expression array 1 according to the manufacturer's instructions with SuperScriptII RT (Gibco BRL, Gaithersberg, MD). The CLONTECH Atlas™ human cDNA expression array comprises a positively charged 8 X 12 cm nylon membrane, duplicate spotted with 200-600 bp cDNA fragments representing 588 genes and 21 housekeeping genes or control sequences. The hybridization data were collected with a Molecular Dynamics Phosphoimager™ (Sunnyvale, CA). PCA yielded three principal components, which are interpreted as differences in expression due to hsp27 overexpression alone, differences in expression due to doxorubicin treatment alone, and finally the combined differential effects of doxorubicin treatment and hsp27 overexpression.

Genes identified to be altered due to hsp27 overexpression included hsp27 itself which was contained on the array (an important positive control for the methodology), and the proto-oncogene tyrosine kinase c-yes of the src family. Unlike our experience with the DD technique, Dr. Hansen has been able to confirm expression predicted by the array with Western immunoblot analysis. She found that c-yes was significantly suppressed in the hsp27-overexpressing cells. We are currently evaluating the role of yes

in the drug-resistant growth of these cells, and this work will continue as the basis for a grant application which Dr. Hansen will submit in the next year as she advances to a junior faculty position.

There were several genes which were altered due to doxorubicin treatment. These included the MAPP kinase erk2, activin, and several integrins. These genes however, are not our first priority and have not been further examined. There also were genes identified which were differentially altered due to hsp27 overexpression and drug treatment. We are continuing to examine these, and they include RhoA which is known to be important for hsp27 effects in some cell types, the RAD8 helicase, and DNA helicase II. Also important is the identification of DNA topoisomerase II, the cellular target for doxorubicin, in this group of differentially affected genes. We found that hsp27 overexpression decreased Topoisomerase IIα and ß expression, and that doxorubicin increases Topoisomerase II expression in control cells, but not in transfectants. These results have thus far lead to one submitted manuscript currently out for review (13), which is included in the Appendix. This discovery also encouraged us to examine the role of specific genes known to be involved in drug-induced apoptosis as will be discussed in our second Approach to Task 2.

Second Approach

Another approach we have undertaken is to examine known pathways important in the processes in which hsp27 expression has been implicated. The first of these pathways, programmed cell death, is an important determinant of a cell's response to drugs. We found that drug-induced apoptosis was inhibited in our transfectants. To then determine which cell death pathway might be influenced by hsp27 expression, we first analyzed the bcl-2 family of proteins that are clearly recognized as important mediators of apoptosis. We utilized Western blot analysis to determine the levels of various family members in our transfectants. Levels of the pro-apoptotic proteins, bak, bcl-X_S and bax were unaffected by hsp27 overexpression. Levels of the survival promoter proteins, bcl-

x_L, mcl_1, and bag were similarly unaffected. We did find that the bcl-2 protein was reduced approximately 2-fold in our transfectants. However, since apoptosis is inhibited in these cells and the bcl-2 protein is an inhibitor of apoptosis, its reduction in the transfectants is not consistent with it playing a role in the phenotype that we see. We conclude that the bcl-2 family of proteins may not be involved in the reduction in drug-induced apoptosis. However, since targeting of Topoisomerase II is an important step in the initiation of apoptosis (14), decreased topo II expression as we discovered in our microarray analyses discussed above, may protect breast cancer cells against doxorubicin-induced apoptosis. We would like to suggest that hsp27 overexpression results in a decreased ability to activate apoptosis and the cell death program, independent of p53 and bcl-2 in these cells. Hsp27 overexpression thus represses specific cell death genes, as yet unknown, necessary for apoptosis along with reducing doxorubicin's target, topo IIα and ß. We envision that these studies will help facilitate the identification of novel targets downstream of hsp27's effect for the development of more specific chemotherapeutic agents designed to circumvent hsp27 and specifically launch the apoptotic machinery of the cell. These results have been recently submitted (13), and again this manuscript is included in the Appendix for review of detailed Experimental Methods and Figures.

Since we have also determined that hsp27 overexpression increases the *in vitro* invasive properties of breast cancer cells (4), we have most recently begun to examine pathways involved in cellular invasion. There is a known association between the matrix metalloproteinases and the invasive or metastatic phenotype. Our preliminary data suggests that matrix metalloproteinase 9 protein (MMP-9) is upregulated in our transfectants. This was observed using a Zymogram where one can assay for the release of typeIV collagenase/gelatinase activity into the conditioned media. This result was recently presented by Dr. Hansen at an international meeting on metastasis (12). Dr.

Hansen will continue to pursue this lead, and will attempt to obtain funding to continue this area of research.

Task 3: To Interfere with hsp27 Expression

We identified and cloned a putative transcription factor that binds to a unique sequence in a region of the hsp27 promoter that was responsible for the majority of basal promoter activity. A partial cDNA clone of 0.9 kb was first isolated by screening an MCF-7 expression library with the hsp27 promoter fragment (-99 bp to -15 bp), followed by the isolation of a full-length cDNA of 1.6 kb from another breast cancer cDNA library. We decided to call the protein HET (130 kDa), for hsp27-ERE-TATA binding protein, since it binds a 1/2ERE-TATA site in the hsp27 promoter. Searching of GENEbank led to the discovery that 1.2 kb of 1.6 kb from our HET clone has 100% homology to a scaffold attachment factor, called SAF-B (15). SAF-B was cloned from a HeLa cell cDNA library. The homology between HET and SAF-B leads us to hypothesize that HET is a nuclear matrix protein. This is exciting because of the increasing realization over the past few years of the importance of higher order structure (e.g. chromatin structure, nucleosomes, and the nuclear matrix) in transcriptional regulation. The nuclear matrix is composed of a number of proteins which provide anchor sites for DNA attachment. These proteins can serve a variety of functions. They may be transcription factors that directly interact with DNA at promoter/enhancer/silencer sites, and/or may be part of the basal transcription machinery (16). They may be structural components which create the right "environment" for transcription (17). They may also indirectly regulate transcription by regulating other transcription factors (18). We were able to show that HET is localized in the nuclear matrix in various breast cancer cell lines. Furthermore, in transient transfection assays using hsp27 promoter-luciferase reporter constructs, HET overexpression resulted in a dose-dependent decrease of hsp27 promoter activity in several cell lines. These results

have been published (19), completing our major goal of identifying regulatory factors which we can utilize to interfere with hsp27 expression.

Another approach to interfering with hsp27 expression is a pharmacological as opposed to a molecular approach described above. To this end, we explored the ability of different agents which inhibit the heat-induced synthesis of heat shock proteins, to inhibit hsp27 expression in breast cancer cells. But a major obstacle to regulating hsp expression has been the lack of a specific inhibitor. Initially, the flavonoids were reported to specifically inhibit hsp synthesis. Since then the mechanism of action of these compounds has been studied intensely, in the hope that they might be used as sensitizing agents in combination chemotherapy We discovered that while quercetin inhibits the heat-induction of hsps in MDA-MB-231 breast cancer cells, it does not inhibit binding of the regulatory factor, heat shock transcription factor (HSF) to their target DNA. Furthermore, while quercetin inhibits HSF2 expression, it only minimally reduces HSF1 expression. In contrast, quercetin inhibits both HSF DNA-binding activity and the expression of HSF2 and HSF1 in non-breast cells, such as HeLa. Our studies suggest that quercetin functions in a cell-type specific manner, regulating HSF transcriptional activity, rather than DNA-binding activity in human breast cancer cells. We speculate that the mechanism by which quercetin inhibits hsp expression in breast cancer cells involves regulation of another step in HSF1 activation, since it does not alter DNA-binding activity. An understanding of this mechanism may aid in the screening and design of future, specific therapeutic agents that could be used to inhibit hsps for the treatment of drug-resistant breast tumors. This work has also been published (20), and is included in the Appendix for reference to the detailed Experimental Methods and Figures.

CONCLUSIONS

We have successfully completed all of our original Specific Aims and Tasks as outlined in the fellowship application. We have examined the major regions of the

hsp27 promoter important for its regulation (Task 1). This lead to both the identification of a new transcription factor which interferes with hsp27 expression and to the identification of a novel mechanism for pharmacological manipulation of hsp27 (Task 3). We have also successfully utilized both a screening aproach, and a direct examination of genes important for hsp27's effects (Task 2). This fellowship has resulted in 5 published manuscripts, and 4 abstracts. The most recent work on Task 2 will most probably result in two more publications and will form the basis for an independent research career for Dr. Hansen. Undoubtedly, the funds for this fellowship have been well-spent with much success. It is also conceivable that this line of research will lead to direct translational benefit in the identification of methods to inhibit hsp27 onerous effects in patients.

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Task 2

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PERSONNEL

Dr. Carl Castles, Postdoctoral Fellow (1995-1996)

Dr. Rhonda Hansen, Postdoctoral Fellow (1996-1998)

Appendices

Appendix 1
Reprint: Basal Regulatory Promoter Elements of the hsp27 Gene in Human Breast Cancer Cells

Appendix 2
Reprint: RNA from Air-Dried Frozen Sections for RT-PCR and Differential Display

Appendix 3
Reprint: Application of Differential Display to Cancer Research: Amplification of RNA Isolated from Air-Dried Frozen and Archival Paraffin-embedded Tissues

Appendix 4
Reprint: Novel Nuclear Matrix Protein HET Binds To and Influences Activity of the HSP27 Promoter in Human Breast Cancer Cells

Appendix 5
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Submitted: Hsp27 Overexpression Inhibits Doxorubicin-Induced Apoptosis in Human Breast Cancer Cells

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Basal Regulatory Promoter Elements of the hsp27 Gene in Human Breast Cancer Cells[1]

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The small human heat shock protein hsp27 has been shown to play important roles in diverse cellular processes such as actin polymerization, thermotolerance, growth, and chemotherapeutic drug resistance. Two breast cancer cell lines MCF-7 and MDA-MB-231 were used as a model to study the molecular mechanisms important for basal hsp27 promoter transcriptional activity. A genomic clone containing 1.1 kb of the hsp27 promoter was sequenced and the regulatory elements were characterized. The first 200 bp within this 5′-flanking region holds the majority of the transcriptional activity, according to transient transfection assays using a series of hsp27 promoter deletion fragments in luciferase reporter vectors. The basal activity of this fragment is largely confined to a G/C-rich region containing overlapping SP1 and AP2 transcription factor binding sites.

Hsp27 belongs to the family of small heat shock proteins (hsps) (1). Under normal conditions, high levels of hsp27 are found in normal estrogen target organs of the female reproductive tract, such as the uterus, vagina, cervix, and placenta (2,3). However, lower levels can be detected in several other normal cell types, including testis and smooth muscle tissues (for review see 1). High levels of hsp27 are also found in some human breast tumors and breast cancer cell lines, especially those that are positive for expression of the estrogen receptor (ER) (2). It is unclear why certain cell types express high levels of certain hsps, however it is well known that hsps are induced by a variety of stressful environmental conditions, such as exposure to heat and certain toxic substances (4). Some commonly used therapeutic agents also specifically induce expression of the small hsps (5). Likewise, a variety of different functions have been described for the small hsps, including roles in thermotolerance (6, 7), actin polymerization (8, 9), and protein chaperon activity (10).

It has long been thought that accumulation of hsps is an adaptive response to adverse environmental conditions. A number of studies suggest that hsp27 can protect cells from the cytotoxic effects of chemotherapeutic agents. The first evidence that hsp27 was directly involved in drug resistance was the early work of Huot et al. (11), who stably transfected a human hsp27 cDNA into Chinese hamster ovary cells. Hsp27-overexpressing hamster cells demonstrated increased cross resistance to doxorubicin, colchicine, and vincristine. In our experiments we have observed that heat shock treatment of human breast cancer epithelial cell lines increased their resistance to doxorubicin (12). More recently we have demonstrated that doxorubicin sensitivity of human breast cancer cells can be altered directly by modulating the level of hsp27 (13). These results have encouraged us to explore whether molecular approaches to modulate hsp27 expression could be used to inhibit hsp27-associated drug resistance.

To this aim, we have now used two breast cancer cell lines as a model to investigate the

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[2] To whom correspondence should be addressed.

Abbreviations: ERE, estrogen-responsive element; ER, estrogen-receptor; HSE, heat shock element; HSF, heat shock factor; TK, thymidine kinase.

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mechanism(s) controlling basal hsp27 transcription. Approximately 1.1 kb of 5′-flanking region of the human hsp27 gene was sequenced, and the regulatory elements were characterized. This region of the hsp27 promoter contains several distinct features, including characteristics of housekeeping genes (such as SP1 binding sites), of regulated genes (TATA sequences), of heat-inducible genes (the heat shock element or HSE), and of estrogen-inducible genes (an imperfect estrogen response element or ERE). We have determined that the majority of the hsp27 promoter activity is contained within the proximal 200 bp of this 5′ region.

MATERIALS AND METHODS

Cell culture and transfection. The human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). MCF-7 cells were originally obtained from Dr. Herbert Soule (Michigan Cancer Foundation). These cell lines were maintained as described in (13). For transient transfection assays, 8×10^4 cells were plated in 6-well plates. Transfections were carried out by the Lipofectamine method according to the manufacturer's protocol (Life Technologies, Grand Island, NY). Cells were cotransfected with 1 μg of the different hsp27 promoter fragment-luciferase reporter constructions plus 0.2 μg of an pRSV-βgal control plasmid. The cells were incubated with DNA for 14 h, harvested 24 h later, and then analyzed for β-galactosidase and luciferase activities. Luciferase activity was determined using a commercial kit (Promega Corp., Madison, WS). β-galactosidase activity was assayed to correct for transfection efficiency, and was determined as described (14) using chlorophenolred β-galactopyranoside (CPRG; Boehringer Mannheim, Indianapolis, IN). Estrogen receptor (ER)-associated influence on the transcriptional activity was carried out by cotransfecting 20 ng of a CMV-driven ER expression vector into the ER-negative MDA-MB-231 cells line. For these experiments the cells were kept on phenol red-free Eagle's Medium containing 10% charcoal-treated FBS. Eight hours before harvesting, 10^{-8} M estradiol was added to the cells.

Plasmid constructions. The source of the human hsp27 promoter sequence was a 1105 bp Bgl II/Aat II fragment from lambda 2711 (15). This fragment contains both transcription start sites and extends into the 5′-untranslated leader region of the mRNA. The 3′ Aat II site was converted into a Hind III site by treatment with mung bean nuclease followed by insertion of a synthetic linker, and subcloning into BlueScript KS+ (Stratagene, La Jolla, CA). This fragment was then sequenced to determine an additional kb of upstream sequence beyond that reported (15). Four promoter 5′ deletion fragments terminating at the 3′ Hind III sites were generated by digestion with a 5′ Bgl II (–1090 bp), Bam HI (–722 bp), Pst I (–441 bp), and Eco RI (–210 bp). Each was introduced into the luciferase expression vector pLuc F3 replacing the SV40 promoter. To determine the specific elements in the most proximal region of the hsp27 promoter, three consecutive fragments designated E, F and G were amplified by polymerase chain reaction (PCR) using sense primers 6 (5′-CCTTAACGAGAGAAGGTTCCAGATGAGG-3′), 7 (5′-TGAGGGCTGAACCCTCTT-3′), 8 (5′-CTCAAA-CGGGTCATTGCCAT-3′), respectively, and antisense primer 5 (5′-TGCTCAGAAAAGTGCGG-3′) (the location of the primers is shown in Fig. 1). Hind III sites plus 2 additional nucleotides were added to the 5′ end of each primer for cloning purposes. The purified PCR products were Hind III digested and ligated into the Hind III site of the pGL2-Basic Luciferase vector (Promega). A mammalian expression vector for expressing ER was prepared by subcloning the Bam HI/Eco RI fragment from a previously described ER yeast expression plasmid, YEPE10 (16) into a similarly digested pcDNAI plasmid (Invitrogen, San Diego, CA). The reporter plasmid ERE-tk-luciferase was kindly provided by Donald McDonnell (Duke University) (17).

Western and Northern blot analysis. Cells were mechanically harvested, washed in phosphate-buffered saline, and extracted with 5% SDS as previously described (18). Equal amounts of protein (50 μg) were resolved on 12.5% polyacrylamide gels. Proteins were then transferred from the gel to nitrocellulose membranes and subjected to immunodetection with an hsp27-specific monoclonal antibody (19) and the Enhanced Chemiluminescence (ECL) system (DuPont, Boston, MA). Extraction of total RNA from cells and Northern blot analyses were accomplished by a previously described method (12). Hsp27 (20) and pGAD-28 cDNA clones (21) were used for hybridization probes.

Gel-retardation analyses. For the analysis of AP2 and SP1 binding sites, an hsp27 promoter fragment from –205 to –84 bp was used as a probe in gel-retardation assays. This fragment was amplified by PCR (22) using the following primers: sense 5′-ATTTGCTTTTCCTTAACG-3′ and antisense 5′-CAATGACCCGTTTGAG-3′. These two primers correspond to primers 1 and 3 in Fig. 1. Gel-retardation assays were performed as previously described (23) with nuclear extracts (24). Purified SP1 and AP2 proteins were obtained from Promega (Madison, WI). In some experiments a 100-fold excess of unlabeled oligonucleotides containing SP1-(5′-GATCGAACTGACCGCCCGCGGCCCGT-3′) consensus sequences were added as specific competitors. In other experiments, 50 ng, 250 ng or 1250 ng of antiserum specific for SP1 and AP2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added, and the reaction was incubated for an additional 30 min. The SP1 oligonucleotides were obtained from Promega (Madison, WI), and all the other oligonucleotides were synthesized by Genosys (Houston, TX).

GeneBank accession number. The accession number for the human hsp27 promoter sequence reported in this paper is XO3900.

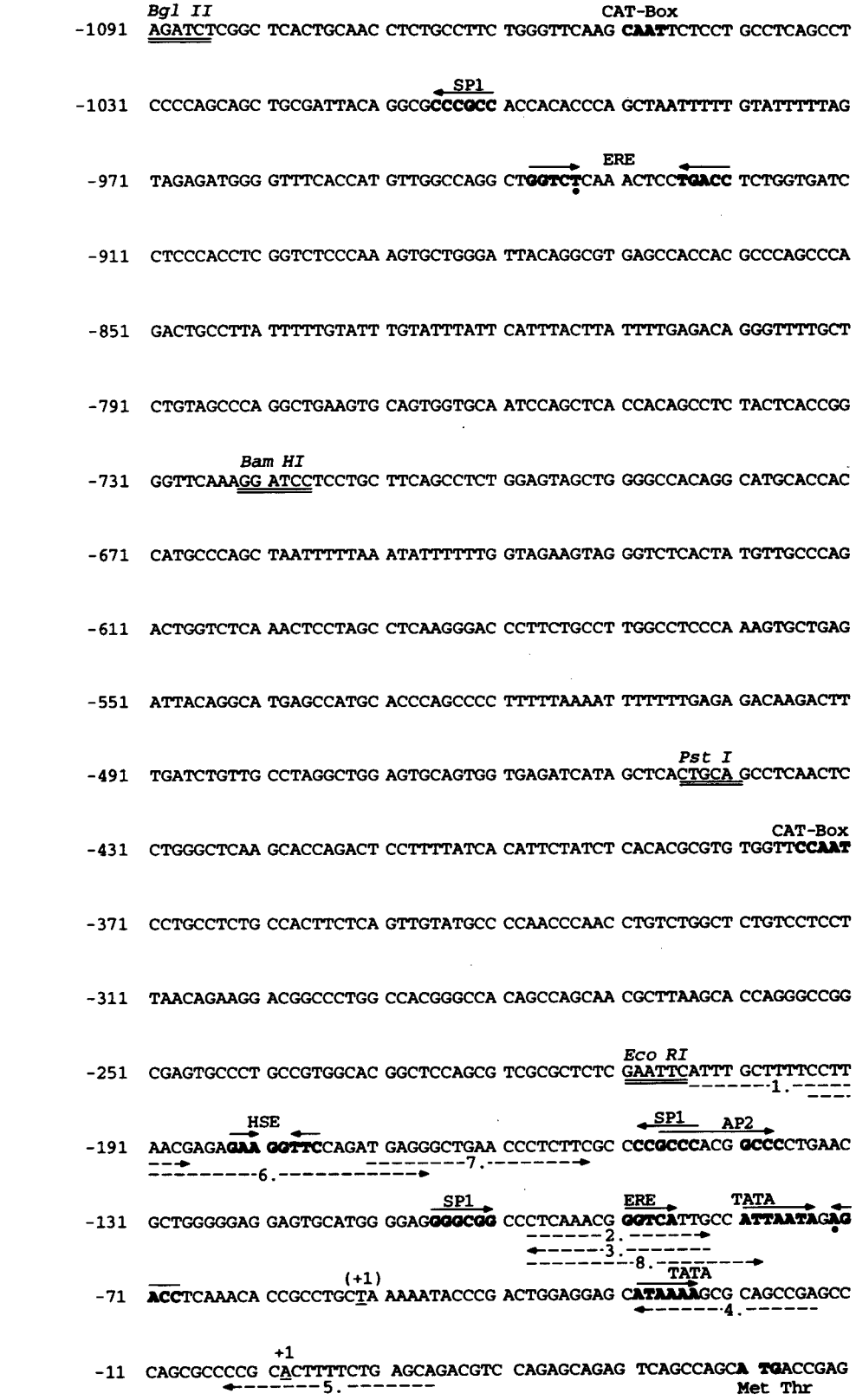
RESULTS

The hsp27 promoter contains multiple putative response elements. Only approximately 200 bp of the 5′-flanking region of the human hsp27 gene has been previously described (15). This 5′-proximal region contains a consensus HSE (GAAnnTTC at −177 to −184), (25, 26), two TATA sequences (ATAAAA and ATTAATA starting at −25 and −75, respectively) (27) upstream from the two mRNA transcription start sites, and finally a putative SP1 binding site (GGGCGG at −102 to −107). We have now determined additional hsp27 gene 5′-upstream sequences and this information along with the previously reported 5′-proximal region is shown in Fig. 1. The hsp27 promoter region contains two potential CAAT elements at −1048 (GCAAT) and at −372 (CCAAT). Approximately 150 bp upstream from the major transcription start site (15), there is an expanded G/C-rich region. This area contains two, partially-overlapping SP1 and AP2 sites in a tight cluster. The SP1 site starting at −145 bp (CCGCCC) is inverted, whereas the one previously reported at −102 bp (15) matches the consensus SP1 sequence (GGGCGG). The AP2 site (28, 29) located within this G/C-rich region is located at −145 to −153 (GCCCCGCCC). Finally, the hsp27 promoter contains elements which resemble ERE half-sites. The ERE half-sites are palindromic; however, the spacer regions between the two half-sites are longer in both cases than the 3 bp spacer found in a perfect consensus ERE (30). Furthermore, both the ERE at −921 (GGTCT-8 bp spacer-TGACC), as well as the ERE at −68 (GGTCA-13 bp-AGACC), have one mismatch each as compared to the consensus vitellogenin A1 ERE (GGTCANNNTGACC). Of interest is the inclusion of a TATA box within the 13 bp spacer of the most proximal ERE half-sites starting at −68. Thus, the promoter of the human hsp27 gene has a compound structure with many potential regulatory elements having features of both housekeeping and regulated genes.

Basal transcription is controlled by sequences in the proximal 200 bp region of the Hsp27 promoter. We chose for study two breast cancer cell lines with different steady state levels of hsp27 protein and RNA. Fig. 2 shows that there are 4 to 5-fold higher levels of hsp 27 protein (panel A) as well as hsp27 RNA expression (panel B) in the MCF-7 cell line as compared to the MDA-MB-231 cell line. First we undertook conventional promoter deletion analyses: Four consecutive hsp27 promoter deletion mutants were generated by restriction digestion and cloned into a luciferase reporter vector (Fig. 3A). We transfected identical amounts of each plasmid into MDA-MB-231 and MCF-7 cells. The results of a representative transient expression assay with these hsp27 constructs is shown in Fig. 3B. In both of the cell lines, the majority of the transcriptional activity was contained within the shortest fragment, the Eco RI (D) fragment. These results suggest that the most proximal region of the hsp27 promoter is the most important region involved in basal regulation.

The results shown in Fig. 3B suggest that a potential negative regulatory element may be contained within the Pst I (C) fragment. We observed that transcriptional activity in MCF-7 cells decreased 2.5-fold when the most proximal hsp27 promoter region was extended by an additional 231 bp from the EcoRI to the PstI site. This result suggests that this region, −210 to −441, may harbor a silencer element. However, there is no similarity between this region and any other region yet described to be active as a silencer element (for review see (31)). To confirm that this region does indeed contain inhibitory activity, we have cloned the Pst I to Eco RI fragment upstream of an SV40 promoter-driven luciferase reporter gene, and found a two-fold reduction of transcriptional activity (results not shown). Inhibition was found to be independent of orientation.

One possible explanation for the differences in hsp27 levels in these two cell lines is the fact that MCF-7 cells contain abundant levels of the estrogen receptor (ER) transcription factor, whereas MDA-MB-231 cells do not. Thus the difference in hsp27 transcriptional activity we observed could be due to ER-mediated transcriptional up-regulation in MCF-7 cells. We therefore tested this hypothesis by transiently cotransfecting MDA-MB-231 cells with an ER expression vector, along with the A through D hsp27 promoter fragments, however no significant increase in activity was


Bgl II
CAT-Box
-1091 AGATCTCGGC TCACTGCAAC CTCTGCCTTC TGGGTTCAAG CAATTCTCCT GCCTCAGCCT
SP1
-1031 CCCCAGCAGC TGCGATTACA GGCGCCCGCC ACCACACCCA GCTAATTTTT GTATTTTTAG
ERE
-971 TAGAGATGGG GTTTCACCAT GTTGGCCAGG CTGGTCTCAA ACTCCTGACC TCTGGTGATC
-911 CTCCCACCTC GGTCTCCCAA AGTGCTGGGA TTACAGGCGT GAGCCACCAC GCCCAGCCCA
-851 GACTGCCTTA TTTTTGTATT TGTATTTATT CATTTACTTA TTTTGAGACA GGGTTTTGCT
-791 CTGTAGCCCA GGCTGAAGTG CAGTGGTGCA ATCCAGCTCA CCACAGCCTC TACTCACCGG
Bam HI
-731 GGTTCAAAGG ATCCTCCTGC TTCAGCCTCT GGAGTAGCTG GGGCCACAGG CATGCACCAC
-671 CATGCCCAGC TAATTTTTAA ATATTTTTTG GTAGAAGTAG GGTCTCACTA TGTTGCCCAG
-611 ACTGGTCTCA AACTCCTAGC CTCAAGGGAC CCTTCTGCCT TGGCCTCCCA AAGTGCTGAG
-551 ATTACAGGCA TGAGCCATGC ACCCAGCCCC TTTTTAAAAT TTTTTTGAGA GACAAGACTT
Pst I
-491 TGATCTGTTG CCTAGGCTGG AGTGCAGTGG TGAGATCATA GCTCACTGCA GCCTCAACTC
CAT-Box
-431 CTGGGCTCAA GCACCAGACT CCTTTTATCA CATTCTATCT CACACGCGTG TGGTTCCAAT
-371 CCTGCCTCTG CCACTTCTCA GTTGTATGCC CCAACCCAAC CTGTCTGGCT CTGTCCTCCT
-311 TAACAGAAGG ACGGCCCTGG CCACGGGCCA CAGCCAGCAA CGCTTAAGCA CCAGGGCCGG
Eco RI
-251 CGAGTGCCCT GCCGTGGCAC GGCTCCAGCG TCGCGCTCTC GAATTCATTT GCTTTTCCTT
1.
HSE
SP1
AP2
-191 AACGAGAGAA GGTTCCAGAT GAGGGCTGAA CCCTCTTCGC CCCGCCCACG GCCCCTGAAC
6.
7.
SP1
ERE
TATA
-131 GCTGGGGGAG GAGTGCATGG GGAGGGGCGG CCCTCAAACG GGTCATTGCC ATTAATAGAG
2.
3.
8.
(+1)
TATA
-71 ACCTCAAACA CCGCCTGCTA AAAATACCCG ACTGGAGGAG CATAAAAGCG CAGCCGAGCC
4.
+1
-11 CAGCGCCCCG CACTTTTCTG AGCAGACGTC CAGAGCAGAG TCAGCCAGCA TGACCGAG
5.
Met Thr

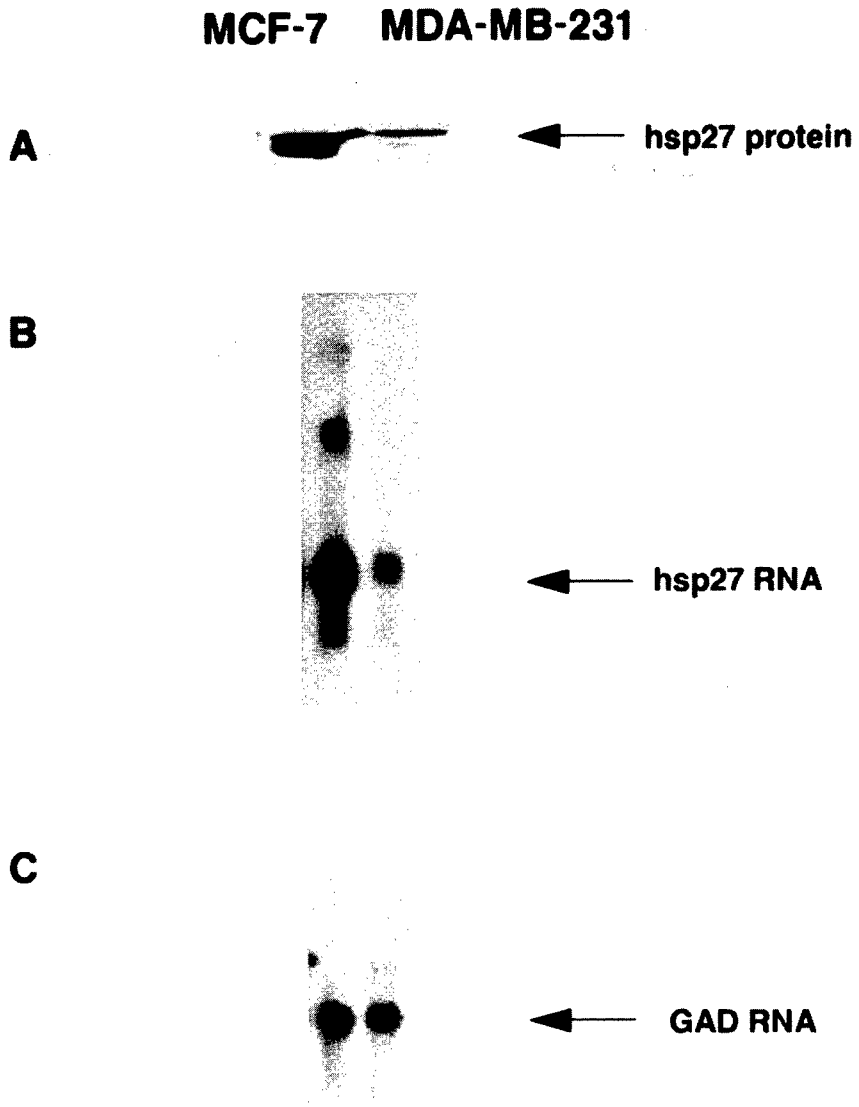


FIG. 2. Hsp27 expression levels in breast cancer cell lines MCF-7 and MDA-MB-231. A) Western blot analysis. B) Northern blot hybridization (hsp27). C) The nylon membrane from B) was reprobed with an pGAD-28 probe.

seen (data not shown). Thus, ER does not appear to be capable of directly transactivating this portion of the hsp27 gene promoter.

The G/C-rich region of the Hsp27 promoter is necessary for activity. As discussed in the previous section, the most proximal hsp27 promoter region (the D fragment in Fig. 3) contained the majority of the transcriptional activity in both MCF-7 and MDA-MB-231 cells. To localize in more detail the specific regulatory elements within this proximal region, we further subcloned +17 to

FIG. 1. Nucleotide sequence of the 1.1 kb 5′ region of hsp27. Bold sequences represent consensus recognition sequences (mismatches are marked with dots), and the arrow denotes the direction of the recognition site. Double underlined sequences are restriction sites used for subcloning of the different promoter regions. Primers used for PCR are indicated by arrows below the sequence.

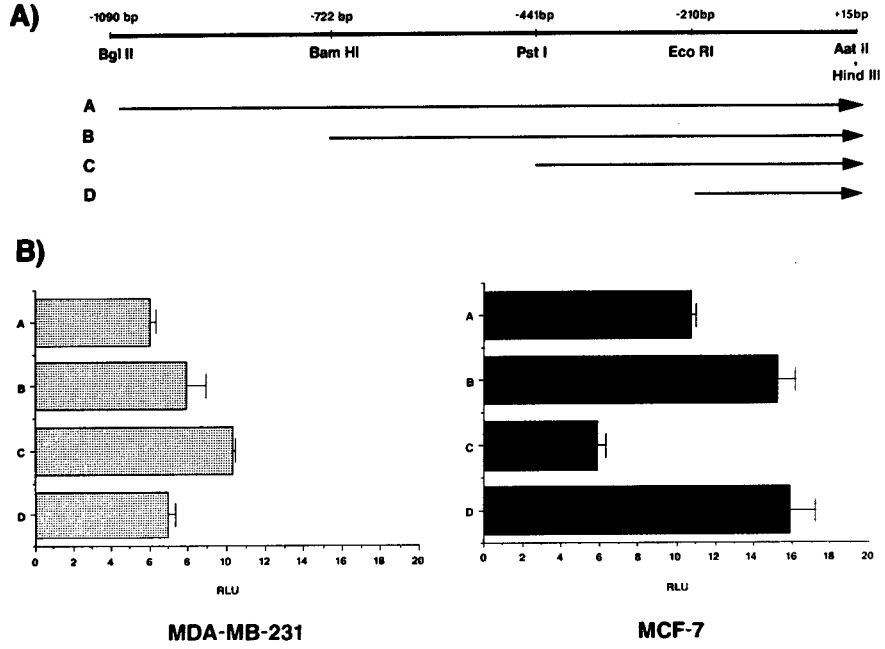


FIG. 3. Transcriptional activity of 5′ deletion mutants of the hsp27 promoter. A) Diagram of the hsp27 promoter deletion constructs. B) Relative luciferase activities (RLU) from experiments performed in duplicate. All transfections were repeated three times and yielded equivalent results.

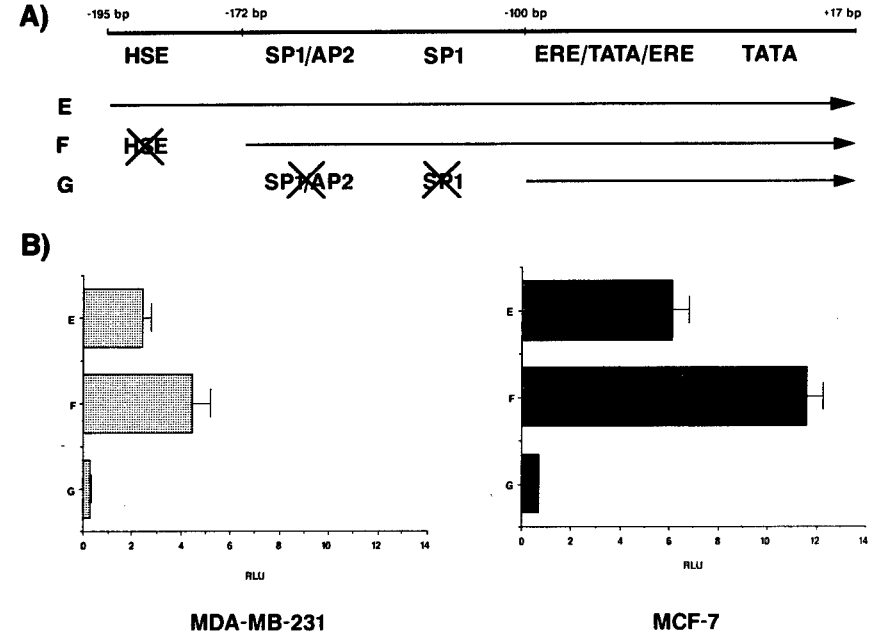


FIG. 4. Transcriptional activity of deletion mutants of the most proximal hsp27 promoter fragment. A) Diagram of the deletions made in the most proximal promoter region. B) Relative luciferase activities from experiments done in triplicate.

−195 bp (Fig. 4A). Three consecutive constructs were prepared: fragment E that essentially corresponds to fragment D in Fig. 3; fragment F in which the HSE has been deleted; and fragment G which eliminates the G/C-rich region containing the putative SP1 and AP2 elements. Interestingly, deletion of the HSE from the hsp27 promoter resulted in an increase in transcriptional activity (compare fragments E versus F in Fig. 4B). What is most apparent from this experiment, however, is that the majority of hsp27 transcriptional activity is lost upon deletion of the region containing the putative SP1 and AP2 sites. To determine whether the putative sites within this G/C-rich region could bind authentic AP2 and/or SP1, we next utilized gel-retardation analyses (Fig. 5) with a probe prepared from this region of the hsp27 promoter. First, commercially-available AP2 and SP1 proteins were used to determine the location of the hsp27 promoter-AP2 (lane 1) and the hsp27 promoter-SP1 (lane 2) retarded complexes in the gel-retardation assay. Nuclear extracts were then prepared from MDA-MB-231 (lanes 3–5) or MCF-7 cells (lanes 6–8), and were found to contain both SP1 and AP2 binding activity. SP1 binding (lanes 4,5, 7,8) was competed with excess nonradioactive SP1 consensus oligonucleotides. These results have been further substantiated with DNase I footprinting of this region (results not shown) and gel-retardation analyses using SP1 and AP2-specific antibodies (Fig. 5, lanes 9–14). The addition of increasing amounts of these antibodies resulted in further upshifts of the specific DNA-protein complexes. These results indicate that this region of the hsp27 promoter is competent for binding of these transcription factors, and furthermore that both factors are expressed in MDA-MB-231 and MCF-7 cells.

DISCUSSION

In this paper we have focused upon the transcriptional regulation of hsp27 in human breast cancer cells. In breast cancer, the emergence of drug resistance during treatment with chemothera-

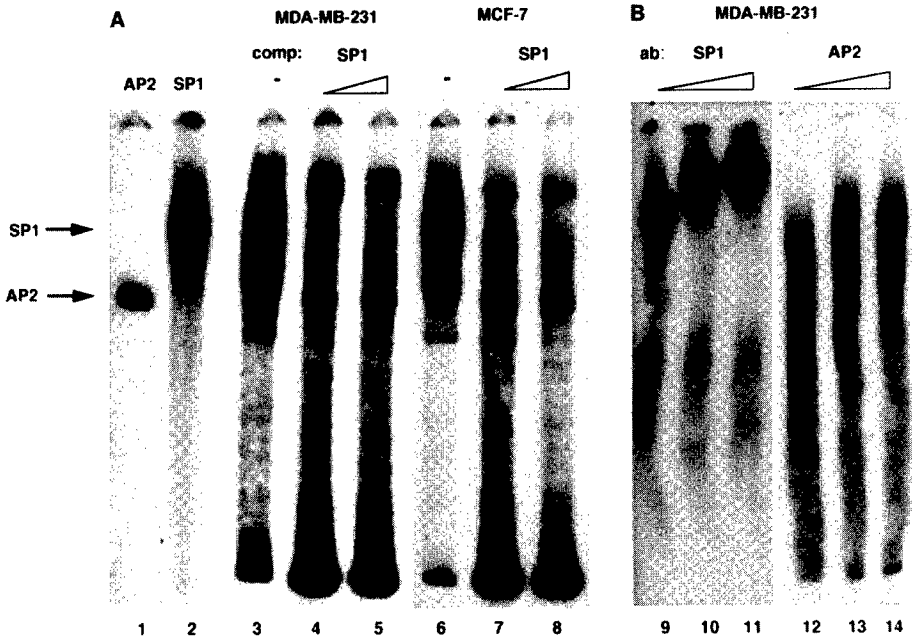


FIG. 5. AP2 and SP1 DNA-binding sites within the proximal hsp27 promoter. An hsp27 promoter fragment from −205 to −84 bp was used as a probe in gel retardation assays. A) In lane 1, purified AP2 protein (25 ng) and in lane 2, purified SP1 protein (30 ng) were added to the reactions. In lanes 3 to 5, MDA-MB-231 nuclear extract was incubated with the probe in the absence (lane 3) or the presence of a 25- and 100-fold excess of unlabeled oligonucleotides. Similar experiments were carried out as described in lanes 6–8, except nuclear extract from MCF-7 cells was utilized. B) MDA-MB-231 extracts were incubated with the probe in the presence of increasing concentrations of SP1 (lanes 9–11) or AP2 (lanes 12–14) specific antibodies.

peutic agents is a major clinical problem. Since hsp27 overexpression confers doxorubicin resistance on breast cancer cells (13), our eventual goal is to identify mechanisms by which we can interfere with this overexpression. We have been using two breast cancer cell lines, an ER-positive MCF-7 cell line and an ER-negative MDA-MB-231 cell line, to study regulation of hsp27 expression. Our previous studies suggest that breast cancer cells can utilize a number of regulatory mechanisms, including estrogen-dependent (32), heat shock transcription factor-dependent, or alternative pathways which are incompletely understood (23), to influence hsp27 levels. Thus the hsp27 promoter is regulated in response to a variety of stimuli in breast cancer cells. Because of the complexity of studying interacting transcriptional mechanisms, we have initially focused on *basal* hsp27 transcriptional activity.

It is known that many regulated promoters are frequently transcribed at basal levels, and we have determined that the hsp27 promoter has a significant level of basal expression in certain breast cancer cell lines. The majority of the hsp27 promoter activity is contained within the 210 bp region adjacent to the transcription start site. Interestingly, this region displays several similarities to elements found in the human hsp70 gene promoter that have been defined to bind specific factors important for basal hsp70 transcription. The proximal 150 bp of the hsp70 promoter contains these elements as the proximal hsp27 promoter, along with two sites which can bind CAAT transcription factor (CTF) (33). In rodent cells the elements required for basal hsp70 expression are the proximal CAT box, an SP1 site, and the TATA box; whereas in human cells, these proximal sequences and additional upstream sequences containing an HSE, an SP1, a CAAT sequence, and an AP2 site are required for basal transcription (34).

The presence of negative regulatory elements in the human hsp27 promoter might explain the observation that the human gene is expressed constitutively when introduced into rodent cells (7). It is possible that the heterologous inhibitory elements are not recognized by the equivalent rodent transcription factors.

The organization of the human hsp27 HSE is very similar to the mouse hsp25 gene, except that the mouse promoter has two palindromic HSE's (35). We can obtain heat shock induction of transcriptional activity in reporter constructions containing the hsp27 HSE (results not shown), thus this single HSE can confer heat inducibility. Interestingly, when the HSE is deleted from the hsp27 promoter, and this HSE-deleted construction is then tested for transcriptional activity, a two-fold increase in basal activity is seen (as shown in Fig. 4). This suggests that an HSE, or factors bound to the HSE, can influence the binding of downstream transcription factors involved in basal promoter activity. A repressing element was also found in the yeast hsp26 gene promoter and it has been proposed that heat-induced derepression plays an important role in activation of the gene during stress (36).

The G/C-rich region containing overlapping SP1/AP2 sites, and an additional downstream SP1 site, is the region most important for basal hsp27 transcription. Little is known about the role of AP2 in specific gene regulation (37, 38), but it is interesting to note that the AP2 transcription factor is induced by retinoic acid treatment in teratocarcinoma cells (39). Since it has been reported that mouse hsp25 can also be induced by retinoic acid treatment (40), it is tempting to speculate that the retinoic acid induction of mouse hsp25 might involve increased AP2 levels. It has also been shown that SP1 can bind to the first SP1 site in the hsp70 promoter, and that this binding stimulates hsp70 transcription *in vitro* (41). Thus, it would not be surprising if SP1 serves a similar important role in hsp27 basal transcription. Since other investigators have successfully targeted an SP1 site to decrease gene transcription in the human Ha-ras promoter, this region might prove to be a similarly useful target to modulate hsp27 expression in breast cancer (42).

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RNA from Air-Dried Frozen Sections for RT-PCR and Differential Display

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Evaluation of in vivo gene expression in specific types of cells is difficult because animal tissues are complex mixtures of many cell types, and RNA extracted from homogenized fresh whole tissue is derived from all cell types present. In an attempt to improve specificity, RNA has been isolated from specific cells microdissected from fixed, paraffin-embedded tissue sections (2,6), but the yield and quality of RNA have generally been suboptimal. Here we report an alternative method for recovering high-quality RNA from specific cells microdissected from air-dried frozen histological sections of unfixed tissue and its analysis by reverse-transcription polymerase chain reaction (RT-PCR) and by the relatively new technique of differential display RT-PCR (DDRT-PCR) (1,3,4).

To prepare air-dried frozen sections, unfixed tissue is quickly snap-frozen or removed from the freezer and adhered to a cryostat chuck. It is critical to avoid thawing in order to inhibit RNase activity. Sections are cut at 20 µm and thaw-mounted on glass slides. The slides are dried in a 37°C incubator for 5 min and either microdissected immediately or stored airtight with a desiccant at < -70°C. Typical equipment for microdissection includes an inverted microscope (with 4× and 10× objectives) and an attached mechanical micromanipulator for holding and manipulating the cutting tool (e.g., a 30-gauge hypodermic needle superglued into a glass microcapillary tube). Specific cells can be visualized without staining the tissue if the microscope contrast is high (e.g., by unfocusing the condenser or using phase-contrast rings) and if an adjacent hematoxylin- and eosin-stained section is used as a guiding template. Alternatively, staining briefly (30 s) with aqueous hematoxylin and quickly re-drying the slide allow for better direct visualization and only slightly decrease the yield of RNA. Accurately separating different types of cells requires familiarity with the histopathological features of the tissue. Approximately 1×10^4 cells are needed to obtain sufficient RNA to run one primer pair in DDRT-PCR or RT-PCR. Thus, dissecting one to several slides may be required depending on the target cell distribution and density. Obtaining sufficient cells can be tedious and time-consuming if the target cells are rare and/or scattered in small groups. Harvesting enough cells can be greatly facilitated by pre-selecting specimens containing relatively large areas of apposed target cells. It is possible to routinely prepare samples enriched to >95% target cellularity, although 100% purity is nearly impossible due to intermingled capillary endothelium, fibroblasts, lymphocytes, etc.

Once the tissue is harvested, RNA can be extracted from air-dried frozen histological sections of breast tissue using methods previously described (6). Following isopropanol-precipitation, sample RNA pellets are washed with 200 µL 80% ethanol, dried under a vacuum for 5 min, or alternatively, the pellets are swabbed with a cotton-tipped appplicator, quickly air-dried and then resuspended in 10 µL of 10× RT buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl and 30 mM $MgCl_2$).

RT of sample RNA was carried out in 0.5-µL thin-wall microcentrifuge tubes (PGC Scientific, Gaithersburg, MD, USA) in a total volume of 96 µL RT buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 3 mM $MgCl_2$) along with 1 mM dNTPs, 0.5 nmol of random hexamer primers and 5 µL of sample RNA (the amount of RNA utilized in the reaction should be empirically determined for different tissues sources). Samples were denatured at 94°C for 2 min, cooled to 42°C for 2 min and then incubated at 42°C for 45 min following the addition of 2.0 U of avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences, St. Petersburg, FL, USA). Following denaturation of the reverse transcriptase at 94°C for 3 min, 0.1 nmol each of sense and antisense primers designed to amplify a small region of the human estrogen receptor (ER) and 2.5 U of AmpliTaq® DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA) were added. The samples were covered with 100 µL of mineral oil, and the reaction tubes were heated at 94°C for 1 min. PCR was then carried out in a Perkin-Elmer cycler using 40 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min. These parameters have proven to be optimum when amplifying this fragment of the ER gene. However, cycling parameters should be optimized for each gene-specific primer pair utilized. These cycling steps were then followed by a single extension cycle of 94°C for 1 min, 55°C for 2 min and 72°C for 7 min. The *Taq* polymerase was then denatured at 98°C for 10 min, and the samples were cooled to 27°C. Ten microliters of each sample were loaded onto a 5% mini-polyacrylamide gel (Bio-Rad, Hercules, CA, USA), separated by electrophoresis and then stained with a 1% solution of ethidium bromide.

As shown in Figure 1, PCR products of the expected size of 170 bp were amplified from four breast tissue samples using oligonucleotide primers specific for separate exons of the ER. It is advisable to choose primers across

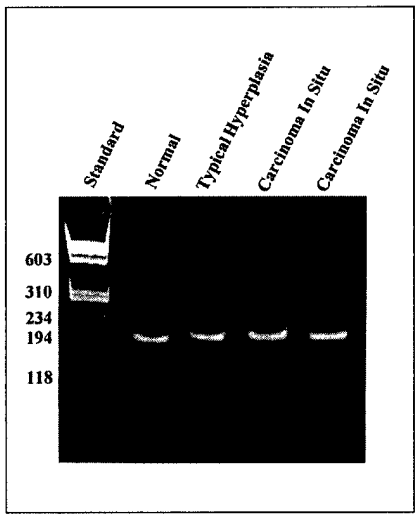

Figure 1. RT-PCR of RNA recovered from air-dried frozen sections. RNA was extracted from histological sections taken from normal breast (lane 1), pre-malignant breast (lane 2) or ductal carcinoma in situ (lanes 3 and 4) and amplified by RT-PCR. Each sample generated a band of the expected size for the PCR product corresponding to a small region of the ER (nucleotides 1542–1712), as well as a larger, less intense band of unknown specificity. Both bands also appeared in an MCF-7 control sample (not shown). Standard = ϕX174/*Hae*III mol. wt. marker (Life Technologies, Gaithersburg, MD, USA).

intronic regions for the gene-specific amplification so that any contaminating genomic DNA present in the RNA preparation will not be amplified. RNA isolated directly from the MCF-7 human breast cancer cell line (5 µg) was amplified as a positive control and yielded identical bands to those seen in Figure 1 (data not shown). Amplifications performed with water in the place of sample, and also reactions lacking reverse transcriptase were carried out as negative controls and gave no amplification products. All PCRs were carried out with equipment and reagents allocated for strict usage in PCR experiments only.

The methodology used for differential display was based on that reported originally by Liang and Pardee (4). RT of each sample was carried out in a total of 50 µL in RT buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 3 mM $MgCl_2$), 50 µM dNTPs, 1 pmol of an oligo(dT) 11-mer antisense oligonucleotide primer ($T_{11}CA$) and either 1.0 µL of sample RNA (again, the amount of RNA added to the reaction should be empirically determined with each tissue source of RNA) or 2 µg of control RNA from MCF-7 cells. Samples were heated to 65°C for 5 min, followed by a 3-min temperature equilibration at 40°C. Five units of AMV reverse transcriptase were added, and the samples were incubated at 40°C for 57 min, followed by denaturation at 94°C for 3 min. The samples were then placed on ice until amplified. We have also found that RT reactions such as these can be maintained at -70°C for up to 6 weeks and they can still be utilized for PCR

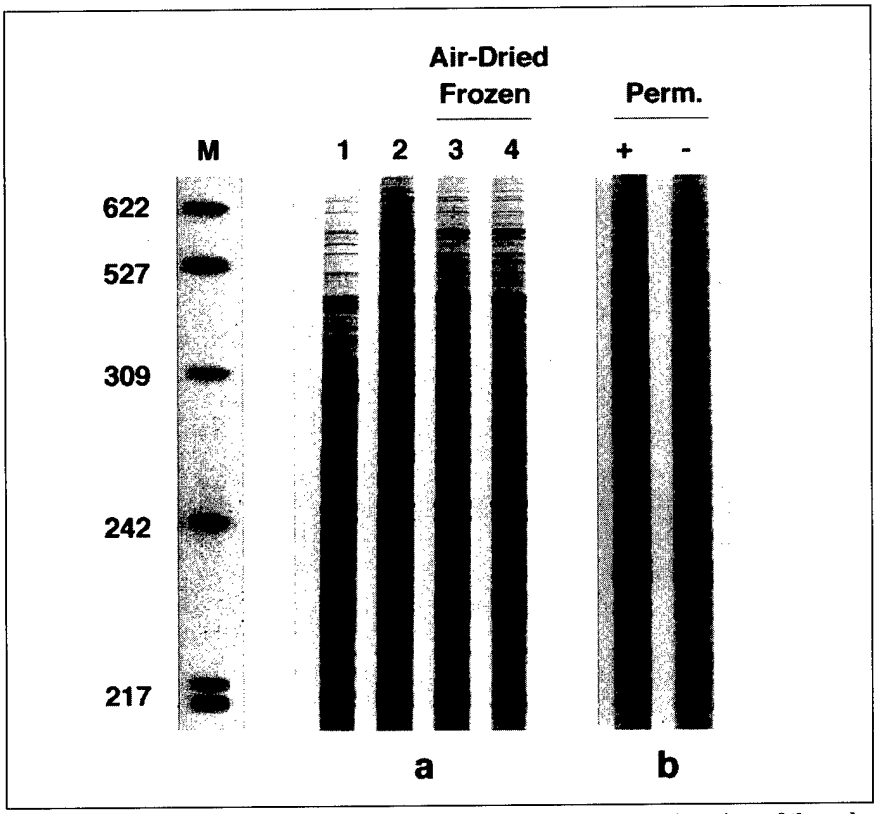

Figure 2. DDRT-PCR results from air-dried frozen and permanent (perm.) sections of the endocervix. Reverse-transcribed cDNA was amplified by PCR from histological sections of endocervix using a $T_{11}CA$ antisense primer and a random 10-mer sense primer as described in the text. In Panel a, the large number of bands in the air-dried frozen samples (lanes 3 and 4) demonstrates that DDRT-PCR can be used for analysis of RNA species as large as 600 bp extracted from tissues of this type. Tissue from the sample in lane 3 was stained with hematoxylin. MCF-7 cell RNA (lane 1) and RNA from a frozen section of endocervix (lane 2) were used as positive controls. In Panel b, DDRT-PCR was performed on RNA recovered from formalin-fixed, permanent (paraffin-embedded) sections (6) of the endocervix with (+) or without (-) hematoxylin staining. Negative controls lacking RT generated no significant banding patterns (not shown). M = *Msp*I-digested pBR322 plasmid (Promega).

amplification for the purposes of differential display (data not shown). PCR amplification of cDNAs was again done in thin-walled microcentrifuge tubes that contained 1.8 µL of PCR amplification buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl and 10 mM $MgCl_2$), an additional 12 µM dNTPs, 4.3 pmol of additional $T_{11}CA$ primer, 5 pmol of a random 10-mer sense oligonucleotide primer and 16 µL PCR-quality water. To this mixture, 7 µCi of [^{35}S]dATP (NEN Life Science Products, Boston, MA, USA), 2 µL of the RT reaction and 2.5 U of *Taq* DNA polymerase were added, followed by a layer of 50 µL of mineral oil. Sample tubes were heated at 94°C for 1.5 min, and amplification was performed for 40 cycles at 94°C for 30 s, 42°C for 1.5 min and 72°C for 30 s, followed by a final extension at 72°C for 5 min, inactivation at 98°C for 10 min and a final cooling to 27°C. Following addition of 5 µL of formamide loading buffer, each sample was heated at 80°C for 3 min, 10 µL loaded onto a 5% Long Ranger™ sequencing gel (AT Biochem, Malvern, PA, USA) and electrophoresed at 70 W for 3 h. Gels were dried under a vacuum at 80°C for 1 h and exposed to X-OMAT film (Eastman Kodak, Rochester, NY, USA) overnight.

As shown in Figure 2a, DDRT-PCR using RNA from MCF-7 control RNA (lane 1), a frozen tissue section of the endocervix (lane 2) and sections of the endocervix that were air-dried at 37°C with or without hematoxylin staining (lanes 3 and 4, respectively) were separated on a 5% acrylamide sequencing gel and exposed to X-ray film. The uniform appearance of bands across all four samples suggests that none of the RNA samples in this experiment have undergone significant degradation and that RNA of sufficient quality for this type of analysis can be recovered from air-dried frozen samples. This is of particular interest, since we had initially expected RNases to greatly impact our ability to recover useful amounts of RNA from air-dried tissue sections, especially those stored at 37°C. In addition, formalin-fixed, paraffin-embedded permanent sections of the endocervix (Figure 2b) also yielded RNA by this published RNA isolation procedure (6), which was readily amplified by DDRT-PCR. However, we have found that fixation should be performed as quickly as possible to avoid RNase digestion. Also, as seen in Figure 2, a and b, we have found that hematoxylin staining does not appear to significantly alter the quality of RNA extracted from archival tissue sections and allows for much easier microdissection. It is very important that the RNA is as free as possible of contaminating DNA. An RNase treatment can be added to the RNA extraction procedure (6) using the suggested manufacturer's procedure (RNase Q™, Promega, Madison, WI, USA) before DDRT-PCR. We also include a reaction lacking RT to ensure that the banding pattern seen is cDNA amplification (data not shown).

Evaluating gene expression by differential display may be relatively simple if the source of RNA is specific and abundant, such as with cell lines. DDRT-PCR is an attractive alternative to subtraction hybridization cloning of RNA amplified from microdissected frozen materials as described by Luqmani and Lymboura (5). In this instance (5), RNA was obtained from breast cancer tissues kept below -20°C during the microdissection. This is in contrast to that reported here, where RNA is obtained from air-dried frozen histological sections, making the actual microdissection process much easier. Animal tissues such as human tumors may contain sufficient RNA for amplification, but it is derived from many cell types, which are present in varying proportions. Specific cells can be obtained from heterogeneous animal tissues by microdissecting them from histological sections viewed under a light microscope. Histological sections are typically prepared from formalin-fixed, paraffin-embedded tissue and stained with hematoxylin and eosin. Unfortunately, the RNA extracted from this type of tissue is sometimes too fragmented and meager for routine differential display. However, we show that adequate amounts of high-quality RNA can be obtained from air-dried frozen histological sections. The relative absence of water in these sections is apparently sufficient to inhibit endogenous RNase activity, making it possible to amplify routinely RNA sequences larger than 600 bp.

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CHAPTER 14

APPLICATION OF DIFFERENTIAL DISPLAY TO CANCER RESEARCH: AMPLIFICATION OF RNA ISOLATED FROM AIR-DRIED FROZEN AND ARCHIVAL PARAFFIN-EMBEDDED TISSUES

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INTRODUCTION

Accumulating molecular evidence in the field of cancer research has demonstrated that the processes contributing to carcinogenesis are largely controlled by the activities of two opposing types of gene products (Cooper, 1995). Oncogenes are genes which produce proteins that have normal cellular functions regulating growth and passage through the cell cycle. However, when expressed inappropriately or when mutated to become more active stimulators of cell growth, oncogene products contribute to the conversion of a normal cell to a cancerous cell. Similarly, tumor suppressor gene (or antioncogene) products are proteins that serve to downregulate cell growth, and if these genes are mutated or lost, the effect is similar to that of removing a brake, allowing a cell to grow and replicate in an uncontrolled fashion. One of the major goals of cancer researchers has been the identification of oncogenes and tumor suppressor genes by a variety of techniques, both to provide an understanding of the mechanisms of the carcinogenic process and to conceivably provide targets for new therapeutic interventions.

The development of the differential display polymerase chain reaction (DD-PCR) method (Liang and Pardee, 1992) has added an important tool to the list of techniques available for the identification of potential oncogene and tumor suppressor gene products involved in carcinogenesis. Products detected in cancer cells but not corresponding normal tissues may be oncogenes, whereas products specifically expressed by normal tissues but not cancer cells are good candidates for tumor suppressor genes. Careful application of differ-

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ential display, coupled with appropriate methods for confirmation of the results of DD-PCR analysis, allows the researcher to screen RNA prepared from diverse sources to identify these genes. RNA for DD-PCR analysis can be obtained from sources as disparate as cancer-derived cell lines, tumor biopsy specimens, or archival blocks of paraffin-embedded specimens.

There are several critical items to be considered for successful application of DD-PCR to cancer-derived materials. Steps must first be taken to enrich target cells as much as possible. This can be done by working with tumor cell lines, which are homogeneous populations of cancer-derived cells, so that differences in cDNAs are attributable to differences in specific manipulations. However, differential cDNA expression can be simply due to differences in cell confluency, accumulation of inhibitory/stimulatory factors in the culture media, or other factors. Thus, even DD-PCR results from a relatively simple system, such as cultured cells, must be interpreted with caution and confirmed with alternative techniques.

When working with animal tissues such as human tumors, the difficulties associated with interpreting differential display results increase dramatically. First, tissue for analysis may be limited since the majority of it may be needed for clinical diagnostic evaluation. However, this problem can be partially circumvented by methods that make use of archival material. Second, all DD-PCR results must be viewed with the caveat that tumor samples are not a homogeneous population of tumor cells, but rather an admixture of cancerous and non-cancerous cells such as normal epithelium, fibroblasts, endothelial cells due to neovascularization, as well as infiltrating inflammatory cells. Therefore, investigators run the risk of having to perform secondary screens on many apparently differentially expressed cDNAs, where in reality, the differences in gene expression are due to varying amounts of contaminating cell types in the original sample. Ideally, isolation of RNA for differential display should be performed only after the investigator has taken steps to isolate the cells of interest (in this case tumor cells) from surrounding noncancerous elements. One method that has proven successful in addressing both of these concerns is microdissection of cancer cells from histological frozen sections that have been air-dried after sectioning to facilitate histiological analysis and microdissection, or thick sections cut from archival paraffin-embedded tissue blocks. These methods form the core of the procedures discussed below.

Finally, it cannot be overemphasized that, in all cases, some additional method should be used to confirm the differences in cDNA expression seen with differential display. Ideally, analysis of expression levels of newly identified gene fragments should be accomplished by probing Northern mRNA blots with a probe prepared by reamplification of the isolated DD band in the presence of a ^{32}P-labeled nucleotide (or nucleotides). Unfortunately, there may be insufficient material available from frozen biopsy specimens to do multiple Northern blots. However, confirmation of DD-PCR bands in clinical materials is feasible with RT-PCR of RNA prepared from frozen sections, or RT-PCR coupled with primer-extension preamplification (PEP) of RNA prepared from paraffin-embedded samples (Zhang et al., 1992), and methods for these are described below.

MATERIALS FOR BASIC METHODS

Preparation of Air-Dried Frozen Histological Sections

Glass microscope slides, 25 × 75 mm, uncoated
Tissue-Tek cryomolds (Miles Inc., Elkhart, IN)
Tissue-Tek OCT compound (Miles Inc.)

Extraction of RNA from Histological Sections

Xylene
80% ethanol
Guanidinium resuspension buffer
Phenol:chloroform (70:30)
Mussel glycogen (5 Prime → 3 Prime, Inc. cat. #5306-851159)
Isopropanol
RQ1-DNase (Promega)
100% ethanol
Phenol/chloroform/isoamyl alcohol
Chloroform/isoamyl alcohol
DEPC treated H_2O
Nutator (Adams Co.)

Differential Display PCR (DD-PCR)

dNTP stock mix
10× RT buffer (↑ Mg)
10× PCR buffer (↓ Mg)
AMV (or M-MLV) reverse transcriptase[a]
T_{11}XX primer at 1 mM
random sequence 5′ 10-mer oligonucleotide primer
^{35}S-dATP, 3000 Ci/mmol, 12.5 mCi/mL (DuPont-NEN cat: #NEG-034H)
Taq polymerase
Mineral oil
10× TBE
50% Long Ranger™ gel solution (AT Biochem)
Formamide loading buffer
pBR-322/*Msp*I radiolabeled marker
Thermal cycler
Sequencing gel apparatus and power supply

RT-PCR Amplification

dNTP stock mix
10× ↑ Mg PCR buffer
5′ and 3′ gene-specific 20-mer oligonucleotide primers
AMV or M-MLV reverse transcriptase[a]
5% acrylamide/TBE minigel

Taq polymerase
mineral oil
Thermal cycler

Primer-Extension Preamplification (PEP)

10× PEP buffer
dNTP stock mix
Random 15-mer primer mix
Pair of gene-specific primers that flank the target sequence (outer 5′ and 3′ primers)
Pair of nested gene specific internal primers (nested 5′ and 3′ primers)
AMV (or M-MLV) reverse transcriptase[a]
Taq polymerase
5% acrylamide/TBE minigel
Mineral oil
Thermal cycler

RECIPES

Guanidinium Resuspension Buffer

6 mg/mL proteinase K
1 M guanidinium thiocyanate
25 mM β-mercaptoethanol
0.5% *N*-lauryl-sarcosine
20 mM Tris-HCL pH 7.5

dNTP stock mix (25 mM each dNTP)

20 μL 0.1M dCTP
20 μL 0.1M dGTP
20 μL 0.1M dATP
20 μL 0.1M dTTP

10× PEP Buffer

100 mM Tris, pH 8.3
500 mM KCl
30 mM $MgCl_2$
10 mM spermidine

10× ↑ Mg PCR Buffer (also 10× RT Buffer)

100 mM Tris, pH 8.3
500 mM KCl
30 mM $MgCl_2$

10× PCR Buffer (↓ Mg)

100 mM Tris, pH 8.3
500 mM KCl
10 mM $MgCl_2$

10× TBE

0.89 M Tris base
0.89 M boric acid
0.02 M EDTA

Formamide Loading Buffer

4 mL formamide
500 μL 10× TBE
0.025 g bromophenol blue
0.025 g xylene cyanol
H_2O to 5.0 mL

STEPS FOR BASIC METHODS

Preparation of Air-Dried Frozen Histological Sections

1. Sample tissue is snap-frozen and immediately adhered to a cryostat chuck.[b]
2. Cut sections at 20 μm and thaw-mount onto glass slides.
3. Desiccate slides in a 37°C incubator for 5 min, then microdissect immediately.[c] Dried slides may be stored in an air-tight container (along with a desiccant) at < -70°C.

Extraction of RNA from Mounted Samples. RNA is extracted from histological sections using methods essentially as described by Stanta and Schneider (1991).

1. Carefully transfer tissue areas microdissected from histological sections into 1.5-mL microcentrifuge tubes.[d] Paraffin-embedded samples can be deparaffinized by adding 1.0 mL of xylene and mixing on a Nutator of 20 mins.
2. Pellet the samples in a microcentrifuge for 5 mins at room temperature, rinse the pellets with 0.5 mL of 80% ethanol (cold), and air-dry for 10 min.
3. Resuspend the dried pellets in 200 μL guanidinium resuspension buffer (6 mg/mL proteinase K, 1 M guanidinium thiocyanate, 25 mM β-mercaptoethanol, 0.5% *N*-lauryl-sarcosine, 20 mM Tris-HCl pH 7.5).[e]
4. Then incubate samples at 45°C for 6 h, followed by a 7-min incubation at 100°C to inactivate the proteinase K.
5. Extract with 200 μL of phenol:chloroform (70:30), mix, then microcentrifuge for 3 min at room temperature, and transfer the aqueous phase to a fresh tube.
6. Add 2 μg of mussel glycogen, an equal volume of room-temperature isopropanol, and precipitate the samples for 1 h at −20°C.[f]

7. Pellet the RNA in a microcentrifuge for 10 min at 4°C, wash the pellet with 500 μL of 80% ethanol (cold), and air-dry the pellet.
8. Resuspended the RNA in 100 μL of DEPC-treated H_2O.
9. Add 1U RQ1 DNase (Promega) to 100 μL of RNA from step 8. Incubate at 37°C for 15 min.
10. Extract once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol.
11. Add 0.1 vol of 3 M NaOAc and 2.5 vol of 100% ethanol. Precipitate for 15–30 min on ice or overnight at −20°C.
12. Repeat step 8.
13. Resuspend the RNA in either 10μL of DEPC-treated H_2O (for RT-PCR), or 100 μL of 1× ↑Mg-PCR amplification buffer (for DD-PCR).

Differential-Display PCR (DD-PCR)

1. Prepare reverse transcription (RT) master mix, say, for 20 reactions:

Add	Final Concentration
100 μL 10× RT (↑ Mg) buffer	1×
5 μL 2.5 mM dNTP mix[g]	12.5 μM
1 μL 1 mM T_{11}XX primer	1 μM
H_2O to 940 μL	

2. Mix 47 μL of master mix with 1–2 μL (0.2–1.0 μg) of RNA solution from step 13.[h]
3. Heat samples at 65°C for 5 min.
   Cool samples to 40°C
   Add 5 units of AMV reverse transcriptase.[i]
   Incubate at 40°C for 60 min.
4. Heat samples to 94°C for 3–5 min to inactivate the RT. Store on ice until ready to perform the DD-PCR, below.
5. Prepare DD-PCR master mix, say, for 20 reactions:

Add	Final Concentration
36 μL 10× RT (↓ Mg) buffer	1×
2 μL 0.5 mM dNTP mix[j]	2.5 μM
1 μL 1 mM T_{11}XX primer	2.5 μM
0.5 μL 1 mM random 10-mer 5′ primer[k]	1.25 μM
15 μL ^{35}S-dATP (3000 Ci/mmol, 12.5 mCi/mL)	~0.5 mCi/mL
H_2O to 350 μL	

6. For each sample, combine in a PCR reaction tube:
   17.5 μL DD-PCR master mix

2 μL RT reaction product
0.5 μL (2.5 units) Taq DNA polymerase

7. Overlay tubes with 50 μL mineral oil.
8. Heat reactions to 94°C for 90 s to ensure complete denaturation.
9. Do 40 cycles of PCR at 94°C for 30 s, 42°C for 90 s, and 72°C for 30 s. Do a single final extension at 72°C for 5 min. To inactivate the remaining Taq enzyme, heat for 10 min at 98°C, and bring samples to 27°C. Samples should be stored at 4°C until ready to load onto the gel.
10. Prepare 5% Long Ranger™/1.0× TBE sequencing gel.
11. Remove PCR samples to fresh tubes[l] and add 5 μL formamide loading buffer.
12. Heat samples and pBR322/*Msp*I radiolabeled marker at 80–85°C for 3 min.
13. Load marker and 10 μL of each sample into 1.0-cm-wide wells on 5% Long Ranger™/1.0× TBE gel.
14. Run gel at 70 W constant power until the xylene cyanol (upper) marker reaches the bottom of the gel.[m]
15. Dry gel onto Whatman 3MM paper under vacuum at 80°C. Expose to film for 16–24 h at room temperature.
16. Bands of interest may be excised and reamplified as described by Liang and Pardee (1992) (see also Chapter 13, this volume).

In the next sections we describe methods to confirm differential expression of the sequences identified using the differential-display procedure. It will be necessary to obtain at least partial DNA sequence of the specific differential display product because the methods described below require sequence specific PCR primers.

DIFFERENTIAL-DISPLAY PROTOCOLS

RT-PCR Amplification of RNA Isolated from Frozen Specimens for Confirmation of Expression of cDNAs Identified by DD-PCR

RT-PCR is performed essentially as described by Fuqua et al. (1990):

1. Set up RT-PCR reactions:
   5–10 μL RNA from extraction procedure (≥ 200 ng RNA)
   10 μL 10× ↑Mg PCR buffer
   3.2 μL dNTP stock mix
   1 μL each 0.1 nmol of 5′ and 3′ gene-specific 20-mer oligonucleotide primers
   H_2O to 98.5 μL

2. Heat to 94°C for 2 min. Cool to 42°C and add 2 units of AMV reverse transcriptase.
3. Heat to 42°C for 45 min. Heat to 94°C for 2 min.
4. Add 2.5 units of Taq polymerase and overlay with 100 μL of mineral oil.
5. Do 35 cycles of PCR at 94°C for 1 min, 55°C for 2 min, 72°C for 3 min. Do a single extension of 72°C for 7 min. Heat 98°C for 10 min. Bring samples to 27°C.
6. Store samples at 4°C.
7. Run 10 μL of each sample on a 5% acrylamide minigel, with standards, to check for expression of candidate cDNAs identified by DD-PCR.

Alternative Protocol for PEP (Primer-Extension Preamplification) of RNA isolated from Archival Specimens for Confirmation of Expression of cDNAs Identified by DD-PCR

1. Set up RT reaction:
   10 μL RNA (all the RNA isolated from a microdissected, 10 μM paraffin-embedded sample)
   5 μL 10× PEP buffer
   1.6 μL dNTP stock solution
   40 μM random 15-mer
   H_2O to final volume of 50 μL
2. Heat to 94°C for 2 min.
3. Add 2 units of AMV Reverse Transcriptase.
4. Heat to 42°C for 45 min and then 94°C for 2 min.
5. Add 2.5 units of Taq polymerase and overlay with 100 μL mineral oil for the initial PCR with random 15-mer oligonucleotide primers.
6. Do 25 cycles of PCR at 94°C for 1 min, 37°C for 2 min, 55°C for 2 min. Do a single extension of 55°C for 3 min. Heat to 98°C for 10 min. Bring samples to 27°C.
7. Set up the first round of gene-specific PCR:
   5 μL from the initial PCR from step 6
   10 μL 10× PEP PCR buffer
   3.2 μL dNTP stock solution
   1 μL 0.1 nmol outer 5′ primer
   1 μL 0.1 nmol outer 3′ primer
   H_2O to a final volume of 99.5 μL
8. Add 2.5 units of Taq polymerase and overlay with 100 μL mineral oil.
9. Do 35 cycles of PCR at 94°C for 1 min, 55°C for 2 min, 72°C for 3 min. Do a single extension of 72°C for 7 min. Heat to 98°C for 10 minutes. Bring samples to 27°C.

10. Set up the second round of gene-specific PCR:
    2 μL of the first round PCR
    10 μL 10× PEP PCR buffer
    3.2 μL dNTP stock solution
    1 μL 0.1 nmol nested 5′ primer
    1 μL 0.1 nmol nested 3′ primer
    H_2O to a final volume of 99.5 μL
11. Add 2.5 units of Taq polymerase and 100 μL mineral oil.
12. Repeat PCR cycles as in step 9.
13. Run 10 μL of each sample from the first- and second-round PCRs on a 5% acrylamide minigel with standards to check for expression of candidate cDNAs identified by DD-PCR.

Notes on the Methods

a. We routinely use AMV reverse transcriptase in our experiments; however, it is likely that M-MLV reverse transcriptase is equally effective. If you decide to use M-MLV reverse transcriptase, alter the reaction buffer to that recommended for M-MLV in order to ensure maximum enzyme activity (see for example, Chapter 13, above).
b. The tissue can also be stored at −70°C for later sectioning. Thawing should be avoided to prevent RNase activity.
c. Typical equipment for microdissection includes an inverted scope and a mechanical micromanipulator for holding and manipulating the cutting tool.
d. This is usually accomplished by scraping with a sterile instrument such as a scalpel blade.
e. Non-paraffin-embedded sections, such as frozen sections or air-dried frozen sections, and paraffin-embedded sections that were not deparaffinized are similarly resuspended in this buffer directly following step 1 of the extraction protocol.
f. For best results, an overnight precipitation is recommended.
g. Do a 1:10 dilution of the dNTP stock to prepare 2.5 mM dNTP mix. Dilute solutions of dNTPs are not stable for storage, and should be made fresh from concentrated stock immediately prior to use.
h. We have had success with as little as 50 ng of RNA per reaction, but for best results we recommend using an amount in the range shown, and using the same amount of RNA in each sample to provide consistency across lanes in the gel.
i. A working stock of RT can be made by diluting the enzyme to 2.5 IU/μL in the RT master mix prepared in step 2.
j. Do a 1:50 dilution of the dNTP stock mix to obtain the 0.5 mM dNTP mix.
k. Less 5′ primer is used to favor the formation of products containing a

$T_{11}XX$ 3′ end. We have found that if equal amounts of primer are used, products containing the random 10-mer at both ends predominate.

l. Sample recovery can be accomplished either by the addition of 75 μl chloroform, which causes the aqueous phase to float to the top, or by carefully pipetting the aqueous reaction mixture from underneath the mineral oil.

m. On a 5% gel, xylene cyanol migrates with an apparent size equivalent to an ~150-base single-stranded DNA. We and others have found that products below this size generally represent contaminating artifacts.

COMMENTARY

Examples of the method of the method are illustrated in Figures 14.1 and 14.2.

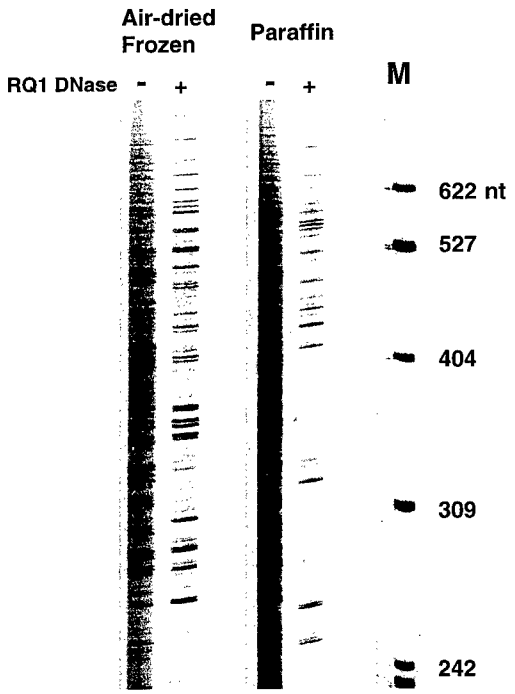

Figure 14.1. Differential display from air-dried, frozen, and paraffin-embedded samples. DD-PCR was performed on RNAs isolated from air-dried frozen sections, or paraffin-embedded permanent sections of normal human cervical tissue. The RNAs were incubated with or without DNase before DD-PCR. Although RNAs can be isolated from both frozen and fixed samples, the best results are obtained from frozen, air-dried, microdissected samples. These figures also emphasize the necessity of DNase-treatment of RNA prior to DD-PCR analysis.

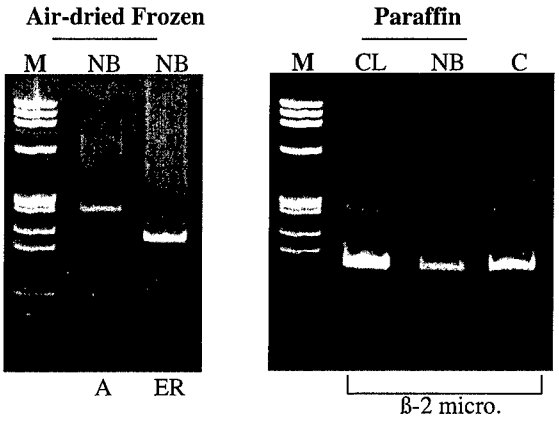

Figure 14.2. RT-PCR of RNA from air-dried, frozen sections or RT-PCR coupled with PEP of RNA isolated from paraffin-embedded permanent sections. RNAs were isolated from air-dried, frozen sections of normal human breast (NB) tissue, or from paraffin-embedded permanent sections of human breast cancer cell line (CL), NB, and normal human cervix (C). The air-dried RNAs were amplified by RT-PCR using either actin-specific (A) primers, and primers to the human estrogen receptor (ER), which is expressed at low levels. The RNAs isolated from the paraffin-embedded sections were amplified by RT-PCR coupled with PEP using primers specific for human β-2-microglobulin. These results demonstrate that confirmation of DD-PCR cDNAs can be performed by RT-PCR methodology using tissue samples microdissected from air-dried, frozen, or paraffin-embedded sections. Neither of these methods, RT-PCR and RT-PCR coupled with PEP, are appropriate methods for quantitation of cDNA expression, but rather are appropriate only for determining whether a candidate cDNA identified by DD-PCR is expressed in specific tissues and tumors.

Critical Parameters

Evaluating gene expression by differential display may be relatively simple if the source of RNA is specific and abundant, such as with cell lines. Mammalian tissues, such as human tumors, may contain sufficient RNA, but they are derived from many cell types that are present in varying proportions. Specific cells can be obtained from heterogeneous animal tissues by microdissecting them from histological sections viewed under a light microscope. Histological sections are typically prepared from formalin-fixed, paraffin-embedded tissue and stained with hematoxylin and eosin (H&E). Unfortunately, the RNA extracted from this type of tissue preparation is usually of insufficient quality for routine DD-PCR. However, adequate amounts of quality RNA can be obtained from air-dried, frozen histological sections. The relative absence of water in these sections is apparently sufficient to inhibit endogenous RNAse activity, making it possible to routinely amplify cDNA's larger than 600 base pairs from this source of RNA.

During microdissection, specific cells of interest can be visualized without staining if the microscope contrast is high (e.g., by unfocusing the condensor or using phase-contrast rings) and an adjacent H&E stained section is used as a guiding template. Alternatively, staining briefly with aqueous hematoxylin

and quickly redrying the slide allows for better direct visualization and only slightly decreases the yield of RNA. Accurately separating different types of cells obviously requires familiarity with the histopathological features of the tissue. About 10^4 cells are needed to obtain sufficient RNA to run one primer pair in DD-PCR or RT-PCR. This may require dissecting one to several slides depending on target cell distribution and density. Obtaining sufficient cells may be tedious and time-consuming if the target cells are rare and/or scattered in small groups. Thus, harvesting enough cells can be greatly facilitated by preselecting specimens containing relatively large areas of apposed target cells. It is possible to routinely prepare samples enriched to >95% target cellularity, although 100% purity is nearly impossible because of intermingled capillary endothelium, fibroblasts, lymphocytes, and other cell types present in tissues.

DD-PCR is best performed using RNA isolated from microdissected frozen, or air-dried frozen-section samples. DD-PCR performed on RNAs isolated from routine clinical paraffin-embedded sections appears to be unreliable, thus making interpretation difficult (although RNA from tissues rapidly fixed in formalin for short periods of time can be of high quality). Furthermore, it must be emphasized that cDNAs differentially displayed in DD-PCR must be confirmed for expression using alternative methods of RNA analysis. RT-PCR of RNA isolated from frozen or air-dried frozen sections is a feasible technique for confirmation using gene-specific primers to the cDNA of interest isolated from the DD-PCR gels. However, the amount of RNA isolated from paraffin-embedded samples precludes direct amplification using gene-specific primers. Instead, one must couple nonspecific amplification techniques, such as PEP, to obtain sufficient cDNA for subsequent PCR with gene-specific primers. Using either of these two techniques, RT-PCR or RT-PCR coupled with PEP, one can quickly confirm differential gene expression of candidate cDNAs in a relatively short timeframe in the specific cells of interest. Microdissection and direct confirmation of gene expression in the target cells of interest is critical for successful application of DD-PCR to clinical samples.

Troubleshooting

The most common problem associated with the recovery of RNA from archival, paraffin-embedded sections is RNA degradation during the tissue fixation and paraffin-embedding procedures. We have found that most of the archival specimens contain partially degraded RNA. Therefore, one must design gene-specific primers that flank the target cDNA sequence such that the target is no greater than 200 base pairs (i.e., $\leq$ 200 bp) in length, with shorter PCR target fragments preferable. Another potential source of degradation is improper storage of the air-dried, frozen mounted samples. The mounted sections, or any microdissected segments from these sections, must be kept dry and used as soon as possible.

Time Considerations

It may take from a few minutes to an hour or more to microdissect histological sections, depending on the amount and distribution of target cells on the slide.

Paraffin-embedded samples take about 50 min to deparaffinize. These samples, along with non-paraffin-embedded sections, are then incubated for 6 h. After approximately 15 min for purification steps, the samples can be precipitated from one hour to overnight. Twenty minutes is then required to prepare the samples to be used in subsequent procedures.

The DD-PCR procedure takes approximately 30 min to one hour to prepare the reverse transcriptase reactions, depending on the number of samples being set up. The RT reaction takes about 70 min, and the PCR cycling reactions an additional 3 h depending on the thermal cycling parameters. It takes about 30 min to prepare the Long Ranger™ gel and one or more hours for the gel to set while the thermal cycler is running. Allow about 30 min to set up the sequencing gel apparatus, to prepare, and then load the samples. The gel will require between 2 and 3 h to run, with approximately one hour to vacuum-dry. Exposure of the gel to film takes 16–24 h (usually done overnight).

Between 30 min and one hour is required to prepare the RT-PCR reactions. The RT reaction takes about 50 min, and after adding Taq polymerase, the PCR cycling requires over 5 hours depending on the model cycler used. For RT-PCR coupled with PEP, one again needs about 30 min to one hour to prepare the reaction mixes. The RT reaction takes about 50 min, and the PEP procedure requires a little over 3 h. The first and second rounds of PCR each require a little over 5 h. The first round can be run overnight and left at 4°C until the next morning, when the second round can be set up and run.

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Novel Nuclear Matrix Protein HET Binds to and Influences Activity of the HSP27 Promoter in Human Breast Cancer Cells

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Abstract Since the small heat shock protein hsp27 enhances both growth and drug resistance in breast cancer cells, and is a bad prognostic factor in certain subsets of breast cancer patients, we have characterized the transcriptional regulation of hsp27, with the long-term goal of targeting its expression clinically. The majority of the promoter activity resides in the most proximal 200 bp. This region contains an imperfect estrogen response element (ERE) that is separated by a 13-bp spacer that contains a TATA box. Gel-shift analysis revealed the binding of a protein (termed HET for Hsp27-ERE-TATA-binding protein) to this region that was neither the estrogen receptor nor TATA-binding protein. We cloned a complete cDNA (2.9 kb) for HET from an MCF-7 cDNA library. To confirm the identity of the HET clone, we expressed a partial HET clone as a glutathione S-transferase fusion protein, and showed binding to the hsp27 promoter fragment in gel-retardation assays. The HET clone is almost identical to a recently published scaffold attachment factor (SAF-B) cloned from a HeLa cell cDNA library. Scaffold attachment factors are a subset of nuclear matrix proteins (NMP) that interact with matrix attachment regions. Analyzing how HET could act as a regulator of hsp27 transcription and as a SAF/NMP, we studied its subnuclear localization and its effect on hsp27 transcription in human breast cancer cells. We were able to show that HET is localized in the nuclear matrix in various breast cancer cell lines. Furthermore, in transient transfection assays using hsp27 promoter-luciferase reporter constructs, HET overexpression resulted in a dose-dependent decrease of hsp27 promoter activity in several cell lines. J. Cell. Biochem. 67:275–286, 1997.

Key words: hsp27 expression; breast cancer; nuclear matrix protein; DNA-binding; promoter; repressor

We have previously established that the small heat shock protein hsp27 plays a role in both growth and drug resistance of human breast cancer cells in culture [Oesterreich et al., 1993]. Supporting this observation, hsp27 has been found to contribute to increased drug resistance in Chinese hamster ovary (CHO) cells [Lavoie et al., 1993], colon cancer cells [Garrido et al., 1996], and testis cancer cells [Richards et al., 1996]. More recently, we have found that elevated hsp27 levels also correlate with increased invasion of human breast cancer cells [Lemieux et al., 1996]. Although many contradictory studies have been published on the prognostic value of hsp27 in various tumors, by analyzing samples from 425 breast cancer patients by immunohistochemistry and 788 samples by Western blot analysis we have found that hsp27 is not an *independent* prognostic marker [Oesterreich et al., 1996b] in breast cancer. However, this large study did show that hsp27 predicts a significantly worse outcome in a subset of estrogen receptor (ER)-positive/

Abbreviations: NMP, nuclear matrix protein; S/MAR, scaffold/nuclear matrix attachment region; hsp, heat shock protein; GST, glutathione S-transferase; ERE, estrogen response element; ER, estrogen receptor; ORF, open reading frame; IF, intermediate filament; ECL, enhanced chemiluminescence

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untreated breast cancer patients. Furthermore, in this study we confirmed previous findings [Dunn et al., 1993; Tandon et al., 1990] that the expression of hsp27 is strongly correlated with the expression of ER in breast tumors. The correlation of hsp27 and ER expression agrees with the finding of estrogen induction of hsp27, which has been described in breast tumors [Seymor et al., 1990] and in breast cancer cell lines [Moretti-Rojas et al., 1988].

Several groups have tried to decrease the expression of hsps in order to circumvent drug resistance in tumors. For example, the antiestrogen toremifene [Mahvi et al., 1996], and the bioflavonoid quercetin [Sliutz et al., 1996], both decrease hsp expression and have been successfully used as chemosensitisers in tissue culture. We have decided to choose a different approach, e.g., by characterizing the hsp27 promoter, with the long-term goal of decreasing expression by targeting its promoter, which might lead to a novel therapeutic strategy in the treatment of breast cancer. While analyzing the hsp27 promoter in detail, we found that most basal transcriptional activity resides in the most proximal region [Oesterreich et al., 1996a]. In this fragment, there is an interesting region of DNA, 70 bp upstream from the start site, consisting of an imperfect palindromic estrogen response element (ERE) that, instead of being separated by the normal 3-bp spacer, is separated by a 13-bp spacer containing a TATA box. Another TATA box 40 bp downstream of this region is the major transcriptional start site, however transcription can also occur from the upstream TATA box [Hickey et al., 1986]. Here we describe the cloning of a protein (HET) binding to this ERE-TATA region (termed HET—Hsp27-ERE-TATA-binding protein), its identification as a nuclear matrix protein, and characterization of its effect on transcriptional activity of the hsp27 promoter in transient transfection assays.

MATERIAL AND METHODS

Cell Culture and Transfection

The human breast cancer cell line MCF-7/MG was originally obtained from Dr. Herbert Soulé (Michigan Cancer Foundation) and has been maintained in our laboratory for the past 14 years. MDA-MB-231 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). MCF-7/MG, MDA-MB-231, and COS cells were maintained as described in [Oesterreich et al., 1996a]. For HeLa cells, the media was not supplemented with insulin or gentamicin sulfate. T47DE and ZR-75 human breast cancer cell lines were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 1% (v/v) L-glutamine, 1% (v/v) glucose, 1% (v/v) penicillin/streptomycin and 5% fetal bovine serum (FBS). Transient transfections were performed as previously described [Oesterreich et al., 1996a], except that Renilla luciferase plasmid (pRL-TK) (Promega, Madison, WI) (50 ng), instead of pRSV-βgal, was cotransfected to correct for transfection efficiency. Briefly, the cells were plated at 2×10^5 cells/well in triplicate in six-well plates, the next day cells were transfected using Lipofectamine (Life Technologies, Grand Island, NY) and OPTIMEM (Gibco-BRL). Cells were transfected with 0.5 μg CF (hsp27 promoter fragment; see below) and with 100 ng HET (MDA-MB-231 and HeLa) and 250 ng HET (T47D, MCF-7/MG and COS cells), respectively. Cells were exposed for 16 h and then incubated in MEM plus 10% FBS for a further 24 h. Cells were lysed and the Dual Luciferase assay (Promega) performed according to the manufacturer's protocol; the values are expressed as relative luciferase units (RLU). The RLU were compared between co-transfection with pcDNAI and HET, respectively, and statistical analysis performed using a two-way analysis of variance (ANOVA) test.

Materials and Plasmids

All materials and chemicals were purchased from Sigma (St Louis, MO) unless otherwise stated. The source of the human hsp27 promoter fragment containing the nonconsensus ERE and the TATA box was a clone originally described in [Fuqua et al., 1989]. The fragment (−99 to −15 bp) was amplified by polymerase chain reaction (PCR), using the following primers: sense 5′-CTCAAACGGGTCATTG-3′ and antisense 5′-TCGGCTGCGCTTTTAT-3′. *Hin*dIII sites plus 2 additional nucleotides (GC) were added to the same primers for cloning purposes. The purified PCR product was digested with HindIII and ligated into pGL2-Basic (Promega) to create the fragment CF. The vector pRLTK (Renilla luciferase gene under thymidine kinase promoter) was also obtained from Promega.

Cloning of HET

An MCF-7 expression library was screened as described [Moretti-Rojas et al., 1988], using a random prime-labeled (Boehringer Mannheim, Indianapolis, IN) hsp27 ERE-TATA promoter fragment. The following modifications were also included in the protocol: the filters were first immersed in 10 mM IPTG for 30 min, followed by denaturation/renaturation steps, including incubation in decreasing concentrations (30 min in 6, 3, 1.5, 0.75, 0.375, and 0.19 M, respectively) of GnHCl in HEPES-buffer (25 mM HEPES, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT). Positive phage DNA was purified, analyzed by dideoxy sequencing of both strands, using the USB (Cleveland, OH) sequencing kit. This partial cDNA (position 680–1,202 bp in the HET sequence) was expressed as a GST-fusion protein to confirm binding to the hsp27 promoter by cloning it into the *Eco*RI site of pGEX-1λT (Pharmacia, Piscataway, NJ). The pGEX-1λT vector clone was used as a negative control in all experiments. GST-HET and pGEX-1λT only were transformed into DH5α and protein was isolated according to the manufacturer's protocol (Pharmacia, Uppsala, Sweden). Briefly, single clones were grown overnight in LB (50 μg/ml ampicillin), the next day an aliquot was grown for 4 h, followed by the addition of 100 mM IPTG, for an additional 1.5 h. The bacteria were pelleted, resuspended in phosphate-buffered saline (PBS) and sonicated for 10 s. After the insoluble material was removed by centrifugation, the supernatant was incubated with glutathione Sepharose 4B for 10 min at room temperature. The beads were then extensively washed with PBS, followed by a 10-min incubation with 5 mM glutathione in order to elute the bound proteins. Aliquots were analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and were also used for gel-retardation assays.

The same partial cDNA clone was then used as a probe to screen another MCF-7 cDNA library (λZap), a kind gift of Dr. Jeffrey Marks (Duke University, NC). Bluescript subclones were excised according to the manufacturer (Stratagene, La Jolla, CA), and analyzed by dideoxy sequencing of both strands using the USB sequencing kit. Since the library was originally constructed by cloning cDNAs into the *Eco*RI site of λZap vectors we excised HET with *Eco*RI and ligated the insert into the eukaryotic expression vector pcDNAI (Invitrogen, Carlsbad, CA).

Southwestern Blotting

Nuclear extracts from MCF-7 cells were separated on a 12.5% SDS–PAGE gel, transferred onto nitrocellulose and the proteins were denatured/renatured by incubating the membrane in decreasing concentrations (10 min in 6, 3, 1.5, 0.75, 0.375, and 0.19 M, respectively) of GnHCl in Z-buffer (25 mM HEPES, pH 7.6, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% NP40, 100 mM KCl, 1 mM DTT, and 10 μM $ZnSO_4$) at 4°C. After blocking for 30 min in Z-buffer and 3% nonfat dry milk, the membrane was incubated with random prime-labeled hsp27 ERE/TATA fragment ($1–2 \times 10^6$ cpm/ml) in Z-buffer and 0.25% nonfat dry milk with 10 μg/ml sonicated calf thymus DNA. Finally, the membrane was washed three times in Z-buffer in 250 mM KCl for a total time of 15 min and exposed to a film.

Gel-Retardation Assay

Gel-retardation assays were carried out as published [Fuqua et al., 1994]. The hsp27 promoter fragment was dephosphorylated and 5′-end labeled using [α-^{32}P]ATP and T4 polynucleotide kinase. The end-labeled fragment was incubated in the presence of 20 μg whole cell extracts [Fuqua et al., 1994], and after a 30-min incubation at room temperature the samples were loaded onto a 5% polyacrylamide gel and electrophoresed for 2.5 h. For supershift experiments, ER-specific antibody mAb H222, which was kindly provided by Dr. G. Greene (Ben May Institute, University of Chicago, IL) was added (40-ng/reaction), and the reaction mixtures were incubated on ice for an additional 2 h.

In Vitro Transcription/Translation

TNT rabbit reticulocyte lysate (Promega) was used according to the manufacturer's recommendation with 2 μg of HET cloned into pcDNAI as a template, and 1 μl of T7 polymerase was added last to the reaction mixture followed by a 90-min incubation at 30°C.

Preparation of HET-Specific Antibodies

Antigenicity and hydrophobicity plots were generated to predict immunogenic peptides for the generation of polyclonal antibodies to HET. A 14-amino acid peptide (A156: C-PEARDSKEDGRKF) was synthesized, coupled to KLH,

and injected into rabbits (Alpha Diagnostic, San Antonio, TX). The resulting antiserum was purified by affinity chromatography. Briefly, the immunogenic peptide was cross-linked through the N-terminal cysteine onto an activated sepharose column (Sulfolink, Pierce, Rockford, IL) according to the manufacturer's protocol; the column was washed with low- and high-salt buffer. After elution of the antibody with 100 mM glycine, at pH 2.5, the antibody was dialyzed overnight against PBS/0.02% NaN_3.

Western Blotting

Protein extracts were resolved on polyacrylamide gels as previously described [Tandon et al., 1989], transferred from the gel to nitrocellulose membranes and subjected to immunodetection [Tandon et al., 1989] with an HET-specific polyclonal antibody (see below) and the enhanced chemiluminescence (ECL) system (DuPont, Boston, MA). Both the HET-specific antibody as well as the antirabbit Ig HRP-conjugated secondary antibody (Amersham, Arlington Heights, IL) were used at a dilution of 1:1,000.

Purification of Nuclear Matrix Proteins

Nuclear matrices were prepared according to methodologies as previously described [Fey and Penman, 1988; Samuel et al., 1997]. Briefly, the samples were resuspended in 10 ml of ice-cold TNM (100 mM NaCl, 300 mM sucrose, 10 mM Tris pH 8.0, 2 mM $MgCl_2$, 1% (v/v) thiodiglycol) with 100 mM PMSF. The cell suspension was homogenized with a Teflon pestle on ice with 0.5% (v/v) Triton X-100 and the nuclei collected by centrifugation. Following centrifugation, the nuclei were resuspended to a concentration of 20 A_{260}/ml in cold digestion buffer (DIG) (50 mM NaCl; 300 mM sucrose; 10 mM Tris–HCl, pH 7.4; 3 mM $MgCl_2$; 1% (v/v) thiodiglycol; 0.5% (v/v) Triton X-100) and digested with DNase I (D5025, Sigma) 20 min at room temperature. Ammonium sulfate (final concentration of 0.25 M) was added and the nuclear matrix (NM-IF) pellet was obtained by centrifugation. The NM-IF pellet was then resuspended in ice-cold DIG buffer with 1 mM PMSF, extracted by adding 4 M NaCl to a final concentration of 2 M, and incubated on ice for 30 min. The NM2-IF pellet was collected by centrifugation and once again resuspended in ice cold DIG buffer, reextracted with 2 M NaCl and centrifuged. The NM2-IF was then resuspended in Disassembly Buffer (8 M urea, 20 mM 2-[N-morpholino]ethane sulfonic acid, pH 6.6; 1 mM EGTA; 1 mM PMSF; 0.1 mM $MgCl_2$; 1% (v/v)-mercaptoethanol), and dialyzed overnight at room temperature against 2 L of Assembly Buffer (0.15 M KCl; 25 mM imidazole, pH 7.1; 5 mM $MgCl_2$; 2 mM DTT; 0.125 M EGTA; 0.2 mM PMSF). The IFs were removed by ultracentrifugation and the resulting supernatant containing NMPs removed. The NMP containing supernatant was then dialyzed against ddH2O for 8 h to reduce the salt concentration and then lyophilized. Lyophilized samples were resuspended in appropriate volumes of 8 M urea, aliquoted and frozen at −20°C. The protocol for Western blot analysis was slightly modified, in that the Tween concentration was increased from 0.1% to 0.2%, and the secondary antibody was used at a dilution of 1:5,000.

GENEbank Accession Number

The accession number for the human HET (Hsp27 ERE-TATA-binding protein) is U72355.

RESULTS

HET Binds to the hsp27 Promoter

To analyze the most transcriptionally active region of the hsp27 promoter [Oesterreich et al., 1996a] in more detail, we first investigated whether protein factor(s) bound to this region. Gel-retardation analysis with an 84-bp promoter fragment containing the ERE-TATA box (−99 bp to −15 bp) and whole cell extracts from MCF-7/MG cells revealed binding of a protein complex (Fig. 1A, lane 3). Since this fragment contains an ERE, separated by a TATA box, we named this protein HET (Hsp27 ERE-TATA-binding protein). As hsp27 has been described as an estrogen-inducible protein, we first excluded the possibility that the factor was the ER by doing supershift experiments using ER-specific antibodies. As a positive control we used a consensus ERE as a probe, and we saw retardation of the probe by ER, which was then further upshifted by adding antibodies to ER (lanes 1 and 2). However, ER antibodies did not upshift the protein complex bound to the hsp27 promoter fragment (lanes 3 and 4). Southwestern blotting of MCF-7/MG nuclear extracts with the same hsp27 promoter fragment (Fig. 1B) indicated binding of two proteins with molecular weights of approximately 120 kDa and 45 kDa. The 45-kDa protein has a molecular weight

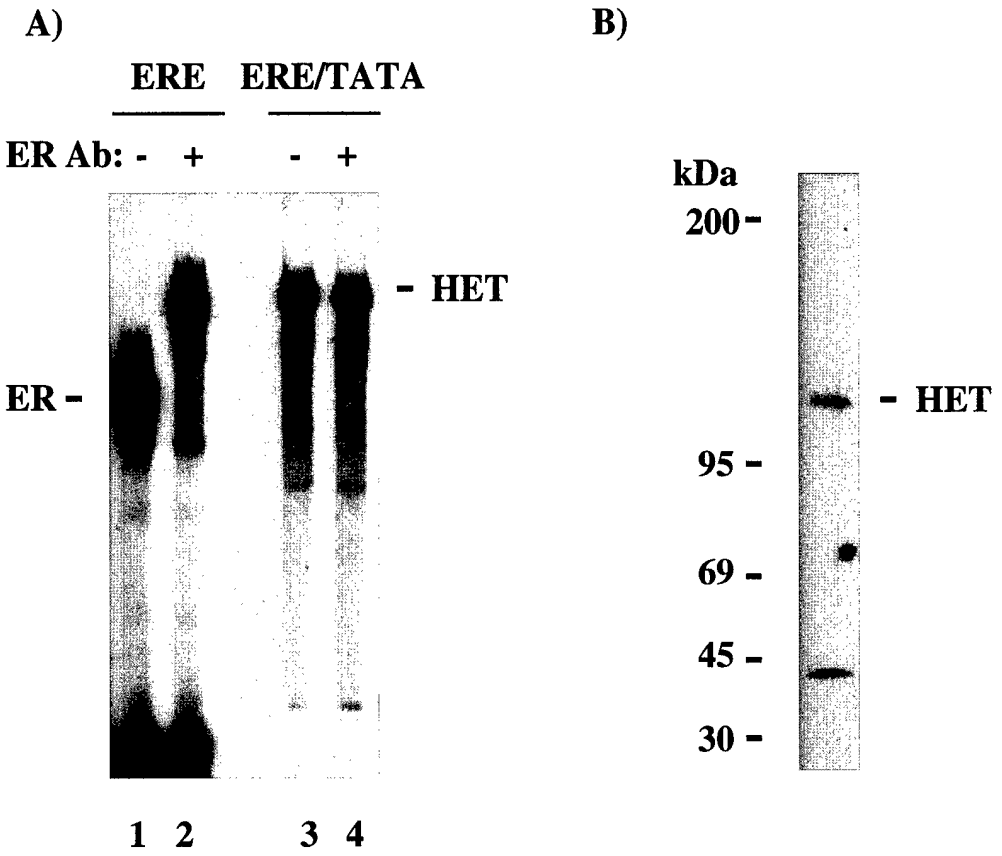

Fig. 1. Identification of HET as an hsp27 promoter binding protein with a molecular weight of approximately 120 kDa. **A:** Gel-retardation assay using a ^{32}P-labeled consensus ERE (**lanes 1 and 2**) and the hsp27 promoter fragment (−99 to −15 bp) containing the ERE/TATA site (**lanes 3 and 4**), respectively, as probes and whole cell extracts from MCF-7/MG cells. ER-antibodies were added to lanes 2 and 4. **B:** Southwestern blot of MCF-7/MG nuclear extract (25 μg) separated by SDS–PAGE, transferred to nitrocellulose, renatured, and probed with the ^{32}P-labeled hsp27 promoter fragment.

consistent with a TATA-binding protein. The difference in size of HET (the 120-kDa protein) as compared to the ER (65 kDa) again indicates that HET is distinct from the ER. Furthermore, we detected binding of HET using extracts of MDA-MB-231 cells (data not shown), which are ER-negative breast cancer cells. We therefore pursued cloning of this hsp27 promoter binding protein.

Cloning of HET as an hsp27 Promoter-Binding Protein

We first isolated a partial cDNA clone (900 bp) by screening an MCF-7 expression library [Moretti-Rojas et al., 1988] with the hsp27 promoter fragment from −99 bp to −15 bp. We then expressed this partial cDNA as a GST fusion protein (Fig. 2A, lane 3), using GST only (Fig. 2A, lane 2) as a negative control. After purification of GST only (Fig. 2A, lane 4) and GST-HET fusion proteins (Fig. 2A, lane 5), we used these extracts in a gel-retardation assay. There we saw specific binding of purified GST-HET to the hsp27 promoter fragment (Fig. 2B, lanes 3 and 4) in a dose-dependent manner, whereas purified GST-alone failed to bind (Fig. 2B, lanes 1 and 2).

We then used this partial clone to screen another MCF-7 breast cancer cell cDNA library (kindly provided by Dr. Jeff Marks, Duke University, NC) and identified an HET clone of 3040 bp in length (GENEbank accession number U72355). The original partial cDNA clone we isolated is identical to position 680–1202 bp in the full-length HET sequence. We confirmed the identity of the HET sequence within the full-length clone by sequencing another four positive clones, which were all overlapping with the sequence from the full-length HET cDNA. The original, partial cDNA clone thus might represent a portion corresponding to a mRNA splicing variant of HET, a hypothesis supported

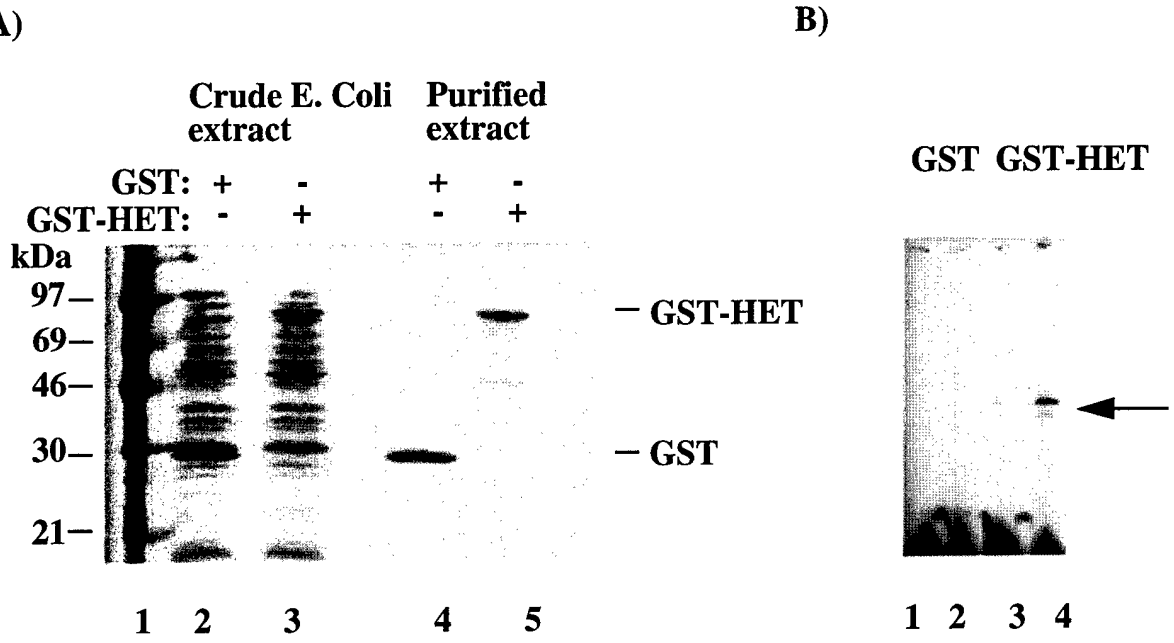

Fig. 2. The GST-HET fusion protein binds to the hsp27 promoter. **A:** Coomassie-stained SDS–PAGE of extracts from *Escherichia coli* expressing GST-HET and GST only (pGEX-1λT) as a negative control, before (**lanes 2 and 3**) and after purification (**lanes 4 and 5**) on glutathione beads. Molecular-weight markers were loaded in **lane 1. B:** 2 and 10 μl of purified GST (**lanes 1 and 2**) and GST-HET (**lanes 3 and 4**), respectively, were then used in a gel-retardation assay using the hsp27 promoter fragment as a probe.

by the appearance of minor bands on Northern blots (data not shown). There is a putative initiation codon at nucleotide 54 in our full-length HET cDNA clone, which shows a weak homology to the translation initiation consensus sequence [Kozak, 1983]. There is also a translation stop codon at nucleotide 2799, which is followed by a polyadenylation signal and a poly(A) tract. A potential open reading frame (ORF) coding for 916 amino acids is present, which results in a predicted molecular weight of approximately 100 kDa for the HET protein. Using PROSITE, we identified several potential phosphorylation, glycosylation, and mysristylation sites, furthermore we learned that the protein contains an unusually high number of charged amino acids (221 negatively charged and 164 positively charged; theoretical pI = 5.05). No classic DNA-binding motif was identified using PROSITE.

To enable further analysis of HET we developed a rabbit polyclonal antiserum. This antiserum recognized HET in whole cell extracts; however, other nonspecific bands were seen (data not shown). After affinity purification (see under Material and Methods), the polyclonal antibody recognized a single band of approximately 120 kDa (data not shown), in agreement with our Southwestern blot analysis (Fig. 1B). The discrepancy between the predicted and the approximate molecular weight by SDS–PAGE, may be a result of post-translational modification. We then used this purified antibody preparation to determine whether it would recognize the HET protein prepared by coupled in vitro transcription/translation. HET cDNA was in vitro transcribed and translated in the presence (Fig. 3A) and absence of [^{35}S]methionine (Fig. 3B), and the protein products were analyzed by SDS–PAGE. [^{35}S]methionine in vitro transcription/translation of HET cDNA resulted in expression of a single high-molecular-weight protein (Fig. 3A, lane 1), whereas no protein was detected with control DNA (vector only) (Fig. 3A, lane 2). The protein product from the unlabeled in vitro transcription/translation were exposed to Western blot analysis using the HET-specific purified polyclonal antibody and, as expected, we detected a band comparable in size to the product from the in vitro transcription/translation in the presence of [^{35}S]methionine (Fig. 3B, lane 1). Again, no band was detected when control DNA was used (Fig. 3B, lane 2). Taken together, these facts strongly indicate that the cloned cDNA encodes the full-length HET protein.

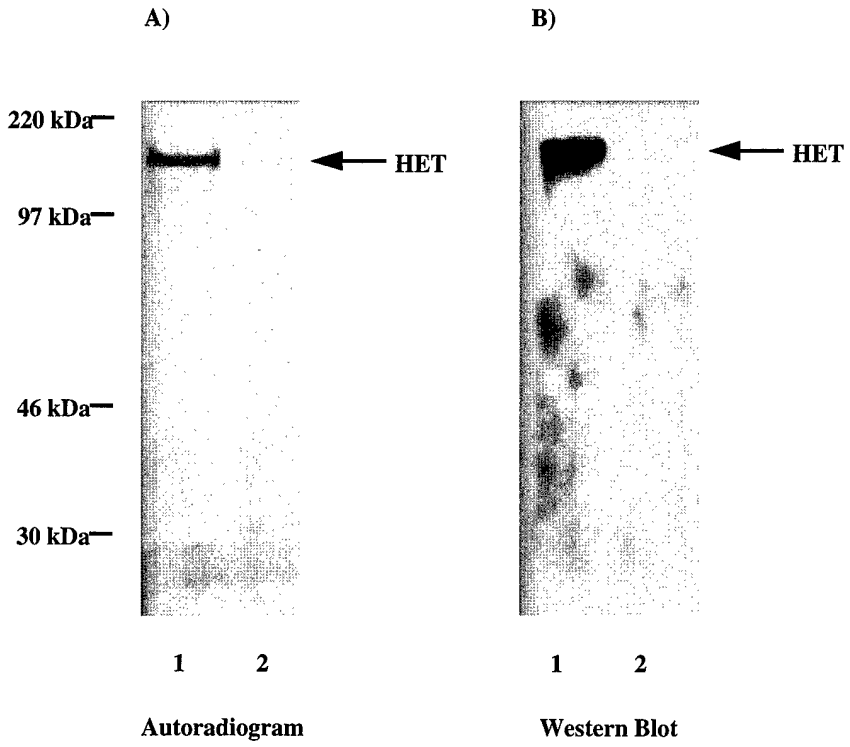

Fig. 3. The HET cDNA encodes for a 120-kDa protein recognized by a purified polyclonal HET-antibody. A) After in vivo excision with helper phage, HET cDNA was recovered in a Bluescript plasmid, which could then be directly used for coupled in vitro transcription/translation analysis. Two μg DNA (HET, **lane 1** and vector only, **lane 2**) were in vitro transcribed and translated using the TnT coupled Reticulocyte Lysate system (Promega) in the presence (**A**) or absence (**B**) of ^{35}S-methionine. The products were then separated by SDS–PAGE, dried, and exposed to a film (**A**), or transferred to nitrocellulose and incubated with 1:1,000 dilution of HET-specific antibody (**B**). The signal was developed using ECL.

HET Is Localized in the Nuclear Matrix

Recently, searching of sequences within GENbank has led to the discovery that HET is almost identical to the scaffold attachment factor SAF-B (GENbank accession number L43631). As a result of a sequencing error at position 344 bp in SAF-B (position 548 bp in HET), a frameshift occurred in the first 100 amino acids, and the 5′ end is apparently missing in the published SAF-B sequence [Renz and Fackelmayer, 1996]. Our HET cDNA has an additional 205 bp at the 5′ end, of which 152 bp code for amino acids. Furthermore, there are 15 mismatches in the 3′ untranslated region comparing HET to SAF-B. SAF-B was recently cloned from a HeLa cell cDNA library [Renz and Fackelmayer, 1996] based on its ability to bind to scaffold/matrix attachment regions (S/MAR), that are AT-rich DNA regions shown to be involved in attaching the base of chromatin loops to the nuclear matrix. Scaffold attachment factors are a specific subset of nuclear matrix proteins (NMP) that specifically bind to S/MAR. We hypothesize that HET is a scaffold attachment factor localized in the nuclear matrix that might also influence hsp27 promoter activity in breast cancer cells. We next tested this hypothesis with the following experiments.

To determine whether HET is localized within the nuclear matrix, various subnuclear fractions from ZR-75, an ER-positive human breast cancer cell line, were isolated as described under Materials and Methods and outlined in Figure 4A. Briefly, cells were homogenized in buffer containing 0.5% Triton X-100 to release lipids and soluble proteins. The nuclei was then digested with DNase I and precipitated with 0.25 M ammonium sulfate to facilitate chromatin removal. The resulting nuclear matrix-intermediate filament (NM-IF) pellet containing nuclear matrix and associated intermediate filaments was then subjected to sequential salt extractions with 2 M NaCl for the further removal of residual histones [Coutts et al., 1996]. The NM2-IF pellet was resuspended in disassembly buffer containing 8 M urea and dialyzed

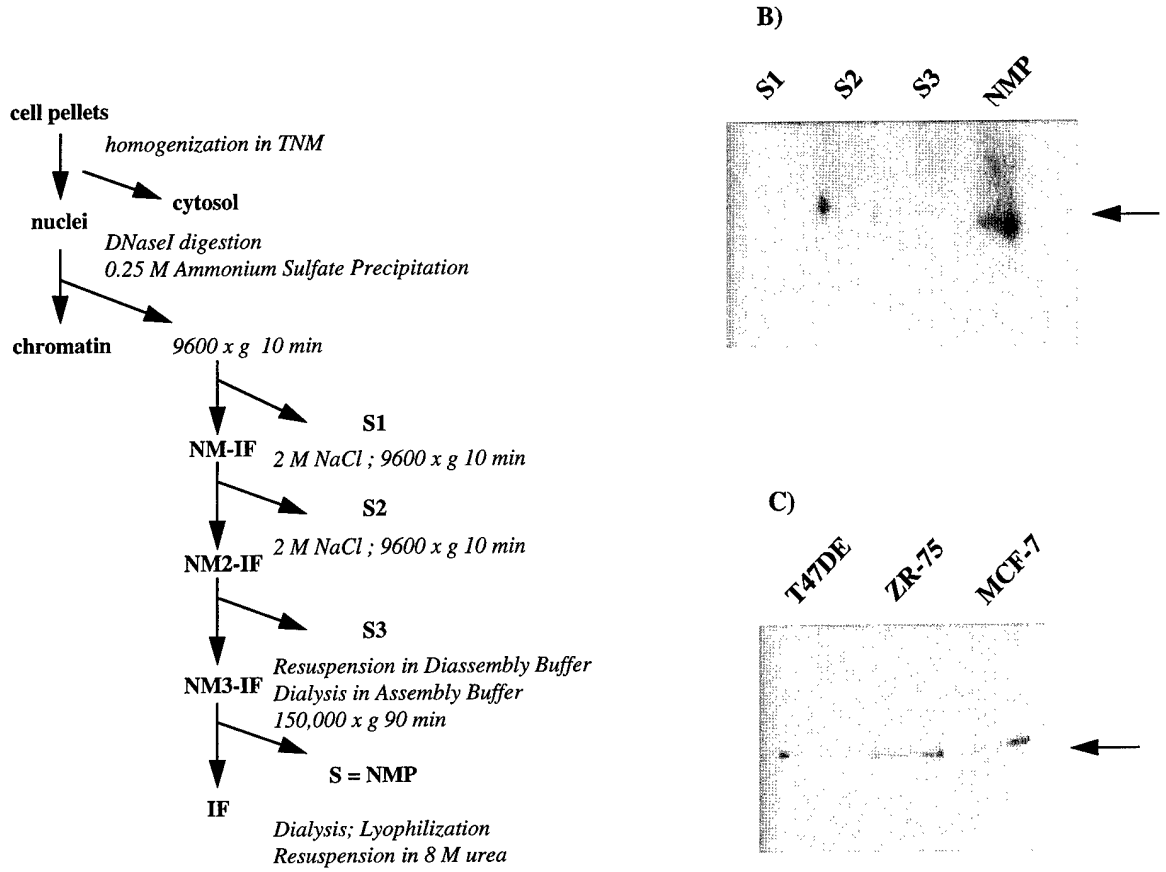

Fig. 4. HET localized in the nuclear matrix. **A:** Diagrammatic representation of the subcellular fractionation scheme. **B:** Twenty µg of protein from various fractions (as indicated) were resolved in each lane by SDS–PAGE, and exposed to Western blot analysis, using HET-antibodies. **C:** Twenty µg of nuclear matrix proteins from various breast cancer cells (as indicated) was resolved in each lane by SDS–PAGE and exposed to Western blot, using HET-antibodies.

against assembly buffer. Dialysis allowed for removal of the urea and for reassembly of IF. The IFs were collected by centrifugation, and the resulting supernatant contained nuclear matrix proteins. Western blotting was then done on all fractions using HET-specific antibodies (Fig. 4B). While there was some HET protein detected in the NM2-IF fraction (S2) (lane 2), most of it was observed to be localized in the nuclear matrix (lane 4). Similar fractions were also prepared from two other ER-positive human breast cancer cell lines (T47DE and MCF-7) to assess whether HET was also localized to the nuclear matrix in these cell lines. Both cell lines were observed to contain similar levels of HET as compared to ZR-75 (Fig. 4C). By contrast, lower levels of HET were detected in the nuclear matrix of MDA-MB 231 cells, an ER-negative human breast cancer cell line (data not shown).

HET Decreases hsp27 Promoter Activity in a Dose-Dependent Fashion

To analyze whether HET would not only bind to the hsp27 promoter but would also influence its transcriptional activity, we performed transient transfection assays with an hsp27 promoter-luciferase reporter construct (called CF) and an expression plasmid containing full-length HET (Fig. 5). CF contains the imperfect ERE and the TATA box region from the hsp27 promoter initially used to clone HET (see under Materials and Methods) and, as shown above, harbors the HET-binding site. The CF fragment displays relatively low basal activity; however, it is about 12-fold higher than that of the pGL2 basic vector alone. We measured the relative luciferase activity of the CF fragment in the presence of either vector alone (pcDNAI) or HET (Fig. 5). In all cell lines analyzed (MDA-

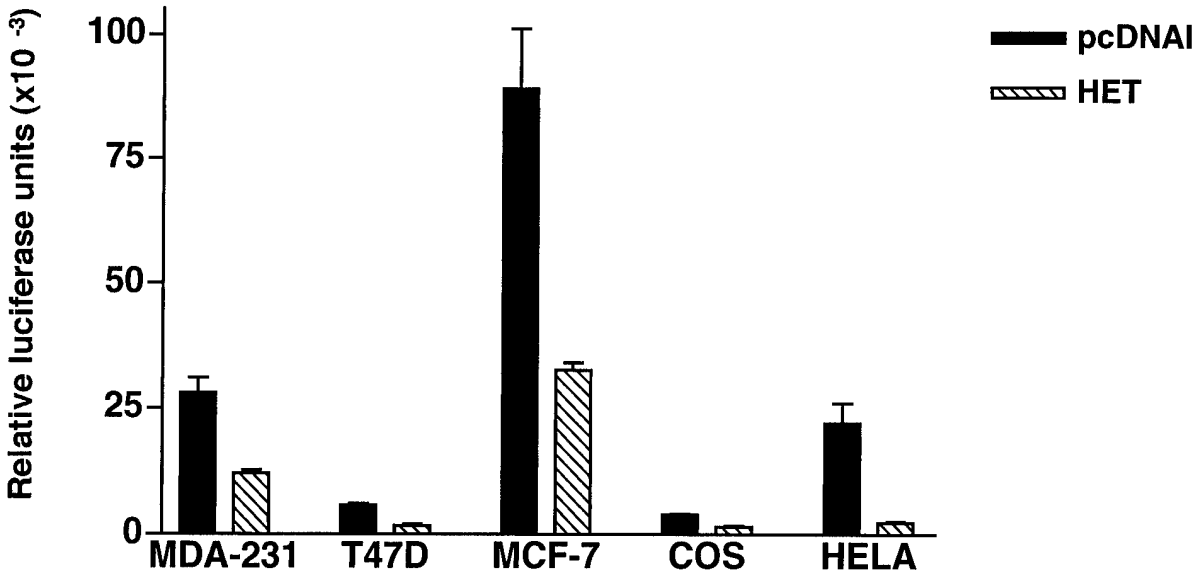

Fig. 5. HET overexpression results in a decrease of hsp27 promoter activity. In transient transfection assays HET was cotransfected with an hsp27 promoter-luciferase construct containing the ERE/TATA site. The luciferase values were corrected for transfection efficiency. Values represent the mean of triplicate wells ± SEM. Significance was calculated by ANOVA.

231, T47D, MCF-7/MG, COS, and HeLa), the co-transfection with HET significantly reduced transcriptional activity of the hsp27 promoter fragment ($P < 0.05$). By contrast, the activity of the Renilla-luciferase construct, which contains a thymidine kinase promoter, and which we used to correct for transfection efficiency, did not decrease after adding HET. To address the question of specificity further, we performed a transient assay using a 1.5-kb IGF-1 gene promoter construct [Adamo et al., 1993], kindly provided by Dr. M. Adamo (UTHSCSA, San Antonio), and pGL2 control, which contains an SV-40 promoter. The activity of the IGF-1 promoter was not affected by HET, and the SV40-promoter was only affected after adding higher concentrations of HET. Therefore, we conclude that HET is able to specifically decrease the activity of the hsp27 promoter in breast cancer cells, however, it may have other targets as described for other NMPs.

DISCUSSION

The small heat shock protein hsp27 is involved in the phenomenon of drug resistance in breast cancer cells [Oesterreich et al., 1993]. Furthermore, overexpression of hsp27 results in increased proliferation in vitro and increased invasiveness in vitro and in vivo [Lemieux et al., 1996]. In accordance with these results, we recently found that hsp27 is a bad prognostic marker in certain ER-positive/untreated breast cancer patients [Oesterreich et al., 1996b]. These recent findings prompted us to study the transcriptional regulation of hsp27 with the long-term goal of intervening with its expression. In a previous report we were able to show that the majority of the promoter activity is in the most proximal 200-bp region of hsp27 [Oesterreich et al., 1996a]; therefore, we have focused first on this region. This region contains an imperfect ERE in which the palindromic half-sites are separated by 13 bp, including a TATA box. In this communication we describe the cloning and characterization of a factor binding to this region, which we have named HET (for Hsp27ERETATA binding).

We first identified HET in gel-retardation assays using the hsp27 promoter fragment as a probe. We then used the same probe to screen an MCF-7 breast cancer cell library, and cloned a 3-kb cDNA coding for HET. This cDNA is almost identical to a recently described human scaffold attachment factor (SAF-B), which was cloned from a HeLa cell cDNA library [Renz and Fackelmayer, 1996]. However, in SAF-B, the 5′ end is missing as a result from a sequencing mistake at position 344 bp, which leads to a frameshift in the first 100 amino acids. Furthermore, our HET cDNA has an additional 205 bp

at the 5′ end. In conclusion, the high sequence similarity between HET and the recently published SAF-B indicates that they are most probably coded for by the same gene.

SAF-B was cloned as one of four proteins (SAF-A through D) that bind to S/MARs. S/MARs are DNA fragments of about 300–3,000 bp, which are AT rich; however, no consensus sequence seems to exist [Gasser et al., 1989]. These regions are involved in the fixation of chromatin to the nuclear matrix and are critical for the organization and function of nuclear chromatin in replication, transcription, and mitosis. Classic AT-rich S/MARs have been proposed to anchor the core enhancers and core origins complexed with low abundancy transcription factors to the nuclear matrix by the cooperative binding of abundant nuclear matrix proteins to S/MARs. This creates a unique nuclear microenvironment rich in regulatory proteins able to sustain transcription, replication, repair, and recombination. S/MARs have also attracted considerable interest in cancer research during the last decade. For instance, cancer researchers have been trying to understand why AT-rich regions are the preferable target for many highly cytotoxic drugs [Woynarowski et al., 1995]. It has become clear that these regions coincide with S/MARs [for review, see Boulikas, 1995] and that the binding of AT-specific drugs to these regions impedes important regulatory functions. Although the hsp27 fragment we used to identify/clone HET is much smaller than the average S/MAR, it is possible that this region is part of a S/MAR. As described for classic S/MAR, this region is very AT rich and harbors a site that is hypersensitive against DNase I, possibly indicating an altered chromatin structure site (S. Oesterreich and S.A.W. Fuqua, unpublished data). Future experiments will be directed at determining whether the hsp27 promoter is indeed a S/MAR and whether it has an appreciable affinity for the nuclear matrix.

S/MAR binding proteins are generally thought to be found within the nuclear matrix. To determine whether HET is localized in the nuclear matrix, biochemical cell fractionation was performed. Subsequently, these fractions were analyzed for the presence of HET using Western blot analysis. As expected, the majority of HET was partitioned in the nuclear matrix with a much lesser amount detected in the NM2-IF fraction. The NM2-IF fraction, obtained after sequential high salt extractions, is considered a nuclear matrix fraction prior to the removal of intermediate filaments [Fey et al., 1984].

Our observation that HET is localized in the nuclear matrix is not in agreement to the study conducted by Renz and Fackelmayer [1996], in which SAF-B was found to be a chromatin protein, but not part of the nuclear matrix. Renz and colleagues explained this observation by suggesting that there are two types of S/MAR DNA binding proteins, which differ in their partitioning upon biochemical fractionation. While we are not in disagreement with this potential explanation, it is also possible that differences in nuclear matrix isolation protocols between Renz and co-workers and our studies have led to this discrepancy. It is important to note that several studies [Dworetzky et al., 1992; Guo et al., 1995; Yanagisawa et al., 1996], suggest that the original nuclear matrix isolation protocol described by Fey et al. [1986] must be modified in order to isolate nuclear matrix from transformed cell lines. This may be one reason why Renz's group failed to detect SAF-B in the nuclear matrix. It is also possible that there are cell type-specific differences in the subcellular localization of HET/SAF-B.

The importance of higher-order nuclear structures, such as NMPs and chromatin, in gene regulation has become increasingly clear; in fact, many NMPs have been shown to be directly involved in transcriptional regulation [Guo et al., 1995; Merriman et al., 1995]. An excellent example is the nuclear matrix protein YY1 [Guo et al., 1995], which can increase or decrease transcriptional activity depending on the target gene and the cell context [Seto et al., 1991; Shi et al., 1991]. The recent discovery that the transcriptional co-activator CBP exhibits histone acetyl transferase activity [Bannister and Kouzarides, 1996] further supports the idea of a direct involvement of higher order nuclear structures in transcriptional regulation. In transient transfection assays, we found that HET decreased the activity of an hsp27 promoter construct, whereas we did not observe a change in activity of a thymidine kinase promoter and IGF-1 promoter in the same assay. However, we did observe an effect with the SV40 promoter (pGL2) and, as with other factors influencing transcription, it is unlikely that HET has only one target gene.

Of specific interest is that the hsp27 promoter region containing the HET binding site and possibly representing a part of an S/MAR also harbors an imperfect ERE. The expression of hsp27 correlates with the expression of ER in both breast cancer cell lines and in breast tumors, and hsp27 has been described as an estrogen-inducible protein. Recently Porter et al. [1996] showed that ER and Sp1 bind cooperatively to the distal half-site of this imperfect ERE, which is in proximity to an Sp1 consensus sequence. This reported binding appears to be very weak, and we would like to hypothesize that HET might stabilize this complex. A similar situation has been described for the vitellogenin B1 promoter, where the relatively weak estrogen response is potentiated through chromatin assembly [Schild et al., 1993]. We are currently testing the hypothesis that in the absence of estrogen, HET leads to a decrease in hsp27 promoter activity; for example, as a result of squelching of the ubiquitous transcription factor Sp1, and in the presence of estrogen, it might lead to an increase in hsp27 promoter activity as a result of stabilization of the Sp1–ER transcription factor complex.

In summary, we have cloned a nuclear matrix protein named HET, which binds to the hsp27 promoter and decreases transcription of hsp27. Before hsp27 was recognized to be involved in drug resistance in breast cancer, the mdr1 gene was characterized as a major player in cellular resistance [Cornwell, 1991]. Recent work by Ince and Scotto [1996] elegantly showed that the promoter activity of the mdr1 gene is strongly influenced by chromatin structure. Nonetheless, the study of higher order structures, such as NMPs, in cancer has been limited to date. However, if indeed the regulation of genes important in breast cancer progression are controlled at least in part by higher order nuclear structures, much more focus should be directed into this area of research.

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Quercetin Inhibits Heat Shock Protein Induction but Not Heat Shock Factor DNA-Binding in Human Breast Carcinoma Cells

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The flavonoid quercetin inhibits the heat-induced synthesis of heat shock proteins (hsps) in a variety of cell lines. To determine whether quercetin could inhibit hsp expression in breast cancer cells, we used the human breast cancer cell line, MDA-MB-231. Treatment of these cells with quercetin decreased the heat-induced synthesis of hsp27 and hsp70. However, inhibition of hsp expression did not correspond with the reduced ability of heat shock transcription factors (HSFs) to bind DNA. Furthermore, while quercetin treatment inhibited HSF2 expression, it only slightly affected HSF1 expression in breast cancer cells. In contrast, quercetin inhibited both HSF DNA-binding activity and HSF expression in HeLa cells. Our studies suggest that quercetin's action is cell-type specific, and in breast cancer cells may involve regulation of HSF transcriptional activity, rather than regulation of its DNA-binding activity.

Organisms respond to environmental stress by reducing the synthesis of some proteins and rapidly synthesizing a number of other proteins (1). These stress-induced proteins, called heat shock proteins (hsps), play various roles in the cell, including chaperoning proteins during synthesis, folding, assembly, and degradation (2). In essence, hsps protect normal cells by providing a defense mechanism against general protein damage. Thus it is not surprising that hsps are relatively abundant in cells and are highly conserved during evolution (2).

In the past few years the role of these proteins in human disease has gained considerable attention (2). Members of the hsp70 and hsp27 families have been implicated in human breast cancer (3, 4). High levels of hsp70 expression in patients with node-negative breast cancer is associated with shorter disease-free survival (5). Initially, hsp27 was thought to also be a significant predictor of worse patient outcome (6, 7). However, a recent report indicates that hsp27 is not a useful independent prognostic marker of disease-free survival in the majority of node-negative patients (8). Hsp27 may play other important roles in the progression of breast cancer. Hsp27 has been associated with drug resistance (9, 10), invasion, and metastatic behavior of breast cancer cells (11). Therefore, hsps present possible treatment targets in breast cancer.

A major obstacle to regulating hsp expression has been the lack of a specific inhibitor. Initially, the flavonoids were reported to specifically inhibit hsp synthesis (12). Since then the mechanism of action of these compounds has been studied intensely, in the hope that they might be used as sensitizing agents in combination chemotherapy (13). The flavonoid quercetin has a wide range of biological activities in mammalian cells; it not only inhibits hsp expression, but also inhibits several protein kinases (14-16). And, not surprisingly, quercetin inhibits the growth of cells *in vitro* (17-20).

In this study, we analyzed the effect of quercetin on hsp expression in human breast cancer cells. We report that while quercetin inhibits the heat-induction of hsps in MDA-MB-231 cells, it does not inhibit binding of HSFs to their target DNA. Furthermore, while quercetin inhibits HSF2 expression, it only minimally reduces HSF1 expression. In contrast, quercetin inhibits both HSF DNA-binding activity and the expression of HSF2 and HSF1 in HeLa cells. Our studies suggest that quercetin functions in a cell-type specific manner, regulating HSF transcriptional activity, rather than DNA-binding activity in human breast cancer cells.

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MATERIALS AND METHODS

Cells and cell culture. HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in MEM complete media supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). MDA-MB-231 cells were also acquired from the ATCC and maintained in MEM supplemented with 10% FBS, 6 ng/ml insulin, and 25 μg/ml gentamicin sulfate. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and periodically tested for Mycoplasma contamination (Bonique Laboratories, Saranac Lake, NY).

Western blot analysis. HeLa and MDA-MB-231 cells were incubated with DMSO as a vehicle or 100 μM quercetin for 6 hours prior to heat shock at 42°C for 1 hour. This treatment was followed by a 4 hour incubation at 37°C, then the cells were mechanically harvested for analysis by Western blot. For analysis of hsp levels, cell extracts were prepared using 5% SDS as previously described (21). Extracts for analysis of HSFs were prepared using a high salt homogenization buffer (20 mM Tris-HCL pH 7.5, 2 mM DTT, 20% glycerol, 0.4 M KCL, 0.3 mM PMSF, 10 μg/ml each of pepstatin, leupeptin, apoprotinin, and antipain) followed by centrifugation at 100,000xg for 5 minutes. In both cases, equal amounts of protein (50 μg) were subjected to SDS-PAGE in 10% polyacrylamide, and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk, incubated for 2 hours at 37°C with primary antibody, then incubated with horseradish peroxidase-coupled secondary antibody. Western blots were developed using the Enhanced Chemiluminescence (ECL) system (DuPont, Boston, MA). Primary antibodies used in this study were anti-hsp27 (Neomarkers, Freemont, CA), anti-hsp70 (Stressgen, Victoria, BC, Canada), anti-HSF1 (22), and anti-HSF2 (22).

Electrophoretic mobility shift analysis. HeLa and MDA-MB-231 cells were incubated with DMSO as a vehicle or 100 μM quercetin for 6 hours prior to heat shock at 42°C for 1 hour. Whole cell extracts were prepared using a high salt homogenization buffer (see above) immediately following heat shock. Gel shifts were performed as previously described (23). Briefly, binding reactions were carried out in a mixture containing 10 μg protein, ^{32}P-labeled oligonucleotide, 3 μg poly(dIdC), 1 mM DTT, 10 mM Tris-HCL pH 7.5, 10% glycerol, 100 mM KCL, 0.3 mM PMSF, 100 ng each of pepstatin, leupeptin, apoprotinin, and antipain in a total volume of 20 μl at room temperature for 30 minutes. For antibody supershifts, HSF1 or HSF2 antibody (22) was added to the binding reaction and the sample incubated at 4°C for an additional 2 hours. Complexes were resolved on 4% nondenaturing polyacrylamide gels at 4°C. Gels were then dried and exposed to film overnight. ^{32}P-labeled, double-stranded hsp27 heat shock element (HSE) (5′- AACGAGAGAAGGTTCCAGATGAGGGCTGAACCCTCTTCGC -3′) (24), or hsp70 HSE (5′- GTCGACGGATCCGAGCGCGCCTCGAATGTTCTAGAAAAGG -3′) (25) oligonucleotides were used as the probes.

RESULTS

hsp27 and hsp70 Expression Following Quercetin Treatment

We examined the effect of quercetin on hsp expression in breast cancer cells using the MDA-MB-231 cell line because these cells have low basal hsp expression which can be induced to high levels with heat shock. Parallel studies were performed with HeLa cells since this cell line has been used in a number of previous studies with quercetin (12, 26). Heat shock treatment of HeLa cells resulted in a 3-fold and 17-fold induction of hsp27 and hsp70 expression, respectively, over basal control levels (Figure 1A, lanes 1 and 2). Incubation with quercetin prior to heat shock inhibited hsp27 and hsp70 induction by 34% and 71% (Figure 1A, lane 3). MDA-MB-231 cells responded in a similar manner (Figure 1B). Heat shock stimulated hsp27 and hsp70 expression by 4-fold and 3-fold, respectively (Figure 1B, lanes 1 and 2), and quercetin treatment reduced both below basal levels (Figure 1B, lane 3). Thus heat-induction of hsp27 and hsp70 were similarly inhibited by quercetin treatment in these cells.

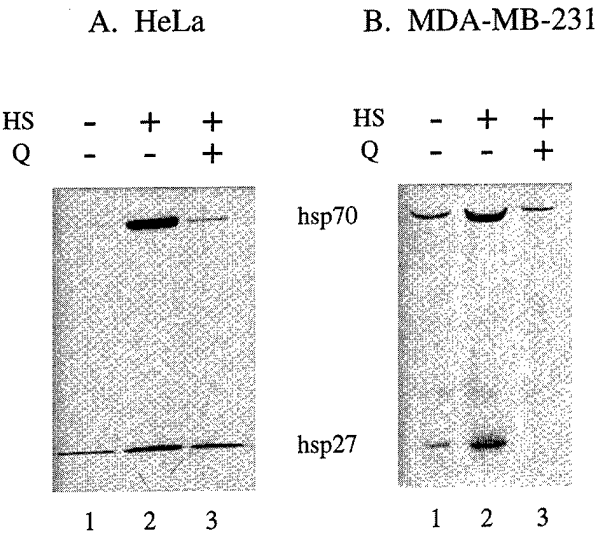

FIG. 1. Hsp27 and hsp70 expression. Western blot analysis of hsp27 and hsp70 in HeLa (Panel A) and MDA-MB-231 (Panel B) cells. Protein extracts from untreated cells (lanes 1 and 2), or cells treated with quercetin (lane 3) were subjected to Western blot analysis using antibodies to hsp27 and hsp70. HS indicates heat shock at 42°C for 1 hour, and Q denotes treatment with 100 μM quercetin. The positions of hsp27 and hsp70 proteins are indicated between Panels A and B.

Effect of Quercetin on Formation of the HSF/HSE DNA-Binding Complex

In HeLa cells, quercetin inhibits the heat-induced binding of HSFs to the HSE (26). To examine the effect of quercetin on formation of the HSF/HSE complex in breast cancer cells, we performed electrophoretic mobility shift assays with both hsp27 HSE and hsp70 HSE oligonucleotides since these HSEs have different sequences. Again, parallel experiments were conducted with HeLa cells. Heat shock stimulated binding of HSFs to both the hsp27 (Figure 2A, lanes 1 and 2) and hsp70 (Figure 2B, lanes 1 and 2) HSEs in HeLa cells. Treatment with quercetin prior to heat shock reduced HSF DNA-binding activity (Figures 2A and 2B, lane 3). However, quercetin treatment in the absence of heat shock did not affect the formation of the basal HSF/HSE complex (Figures 2A and 2B, lane 4). These results correlate with previously published work (26).

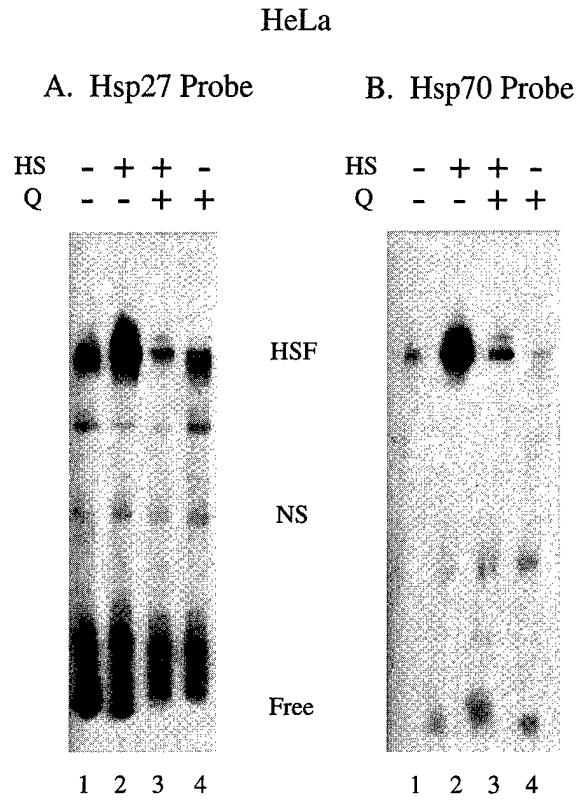

FIG. 2. Formation of the HSF/HSE DNA-binding complex in HeLa cells. Gel mobility shift analysis of the binding of HSF to either the hsp27 probe (Panel A) or the hsp70 probe (Panel B). HS indicates heat shock at 42°C for 1 hour, and Q denotes treatment with 100 μM quercetin. Between Panels A and B, HSF is used to indicate the HSF/HSE complex, NS indicates regions of non-specific binding, and Free marks the location of the free probe.

Heat shock also increased HSF binding to the hsp27 (Figure 3A, lanes 1 and 2) and hsp70 (Figure 3B, lanes 1 and 2) HSEs in MDA-MB-231 cells. Surprisingly, pretreatment of these cells with quercetin did not affect HSF binding to these HSEs (Figure 3A and 3B, lane 3). Similar to HeLa cells, though, quercetin treatment at 37°C had little effect on HSF binding to either HSE (Figure 3A and 3B, lane 4). Therefore, the effect of quercetin on formation of the HSF/HSE DNA-binding complex during heat treatment differs between HeLa and MDA-MB-231 cells. Moreover, duplicate experiments using another breast cancer cell line, MCF-7, yielded results similar to those with MDA-MB-231 cells (data not shown).

To determine which members of the HSF family are contained within the HSF/HSE complex in MDA-MB-231 cells, we performed antibody supershift experiments (Figures 4A and 4B). The HSF/hsp27 HSE complex supershifts with antibodies to HSF1 (Figure 4A, lane 2) and possibly HSF2 (Figure 4A, lane 3). The addition of quercetin did not affect formation of the HSF/hsp27 HSE complex (Figure 4A, lane 4) or the supershifting of this complex (Figure 4A, lane 5). Similar results were obtained using the hsp70 HSE probe (Figure 4B). Therefore, HSF1 is the major component of the complex binding the hsp27 and hsp70 HSEs, while HSF2 is only a very small component of this HSE-binding activity. And, as shown in Figure 3, quercetin treatment did not block HSF1 binding.

HSF1 and HSF2 Expression Following Quercetin Treatment

Previous studies using HeLa cells indicate that pretreatment with quercetin results in a decrease in HSF1 protein levels (26). We also found that heat shock stimulated a 3-fold increase in HSF1 levels (Figure 5A, lanes 1 and 2), and quercetin treatment blocked this induction in HeLa cells (Figure 5A, lane 3). In addition, quercetin reduced the basal level of HSF1 expression by 80% (Figure 5A, lane 4). In contrast, heat shock did not increase HSF1 levels (Figure 5A, lanes 5 and 6), and treatment with quercetin only slightly reduced HSF1 levels in MDA-MB-231 cells (Figure 5A, lanes 7 and 8). Similar results were seen in MCF-7 cells (data

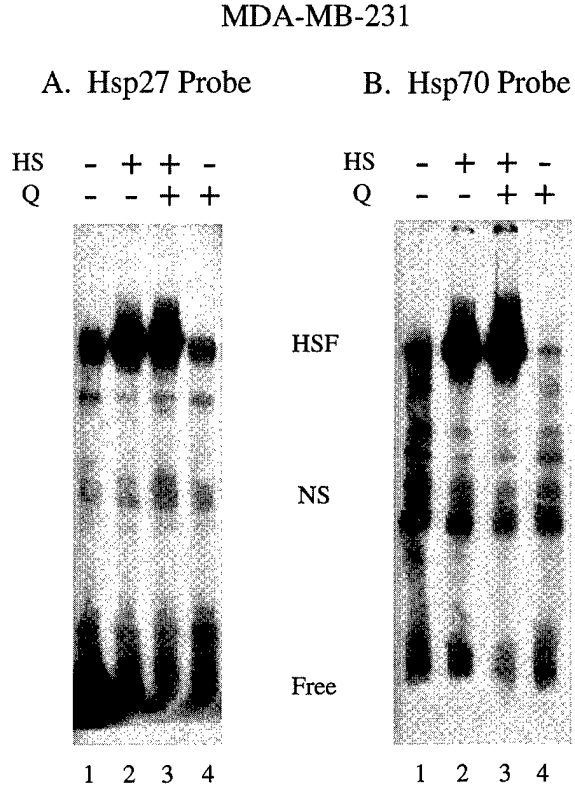

FIG. 3. Formation of the HSF/HSE DNA-binding complex in MDA-MB-231 cells. Gel mobility shift analysis of the binding of HSF to either the hsp27 probe (Panel A) or the hsp70 probe (Panel B). HS indicates heat shock at 42°C for 1 hour, and Q denotes treatment with 100 μM quercetin. Between Panels A and B, HSF is used to indicate the HSF/HSE complex, NS indicates regions of non-specific binding, and Free marks the location of the free probe.

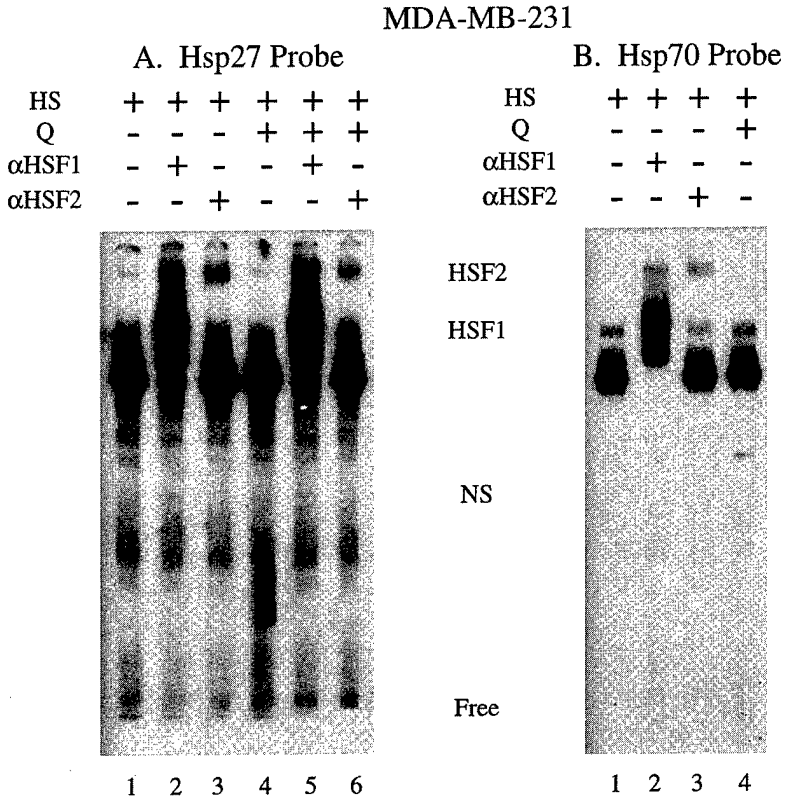

FIG. 4. Antibody supershift analysis in MDA-MB-231 cells. Gel mobility shift analysis of the binding of HSF to either the hsp27 probe (Panel A) or the hsp70 probe (Panel B). HS indicates heat shock at 42°C for 1 hour, Q denotes treatment with 100 μM quercetin, αHSF1 designates antibody to HSF1, and αHSF2 signifies antibody to HSF2. Between Panels A and B, HSF1 is used to indicate the αHSF1 supershifted HSF/HSE complex, HSF2 indicates the αHSF2 supershifted HSF/HSE complex, NS indicates regions of non-specific binding, and Free marks the location of the free probe.

not shown). In contrast, basal and heat-induced levels of HSF2 expression were reduced by quercetin treatment in both HeLa and MDA-MB-231 cells (Figure 5B). Heat shock stimulated a 2-fold increase in HSF2 expression (Figure 5B, lanes 1 and 2, 5 and 6), while pretreatment with quercetin blocked the heat-induction of HSF2 expression in these cells (Figure 5B, lane 3 and lane 7). Finally, treatment under basal conditions also decreased HSF2 expression in both cell lines (Figure 5B, lane 4 and lane 8). Again, similar results were obtained using MCF-7 breast cancer cells (data not shown).

DISCUSSION

Quercetin has been shown to inhibit hsp induction in a number of cell lines; such as colon carcinoma (COLO 320DM, HT-29) (12, 27), HeLa (12), monocyte/macrophages (28), erythroleukemia (K562) (29), and skin keratinocytes (HaCaT) (13). Consistent with these studies, we found that treatment of the breast cancer cell line MDA-MB-231 with quercetin decreased hsp induction. However, this decrease did not correspond to decreased binding of HSFs to the HSE. In these cells, both HSF1 and HSF2 bind the HSEs contained within the hsp27 and hsp70 promoters in the presence of quercetin. This observation contrasts with data indicating that quercetin inhibits HSF DNA-binding activity in COLO 320DM and HeLa cells (26, 30). Interestingly, there are two precedents for our results. In K562 cells, HSF maintains its binding activity following treatment with quercetin, and actually remains bound to the DNA for longer than untreated controls (31). Quercetin treatment also failed to decrease HSF DNA-binding in another cell line, HT-29 (32). In these cells the dissociation of HSF from the HSE was also delayed.

These differences in HSF DNA-binding activity may be due to a number of factors. Quercetin's effect may depend on the type of stress used (heat or chemical), the specific conditions of the stress (length of time and/or temperature), the concentration of quercetin used for treatment, and/or the cell type. Each of the studies cited in the previous section varied in several of these factors. K562 cells, stimulated with PGA_1 instead of heat shock, were treated with a lower concentration of quercetin (30 μM) (31). HT-29 cells were heat-induced at a higher temperature (45°C), then given a higher amount of quercetin (200 μM) (32). COLO 320DM and

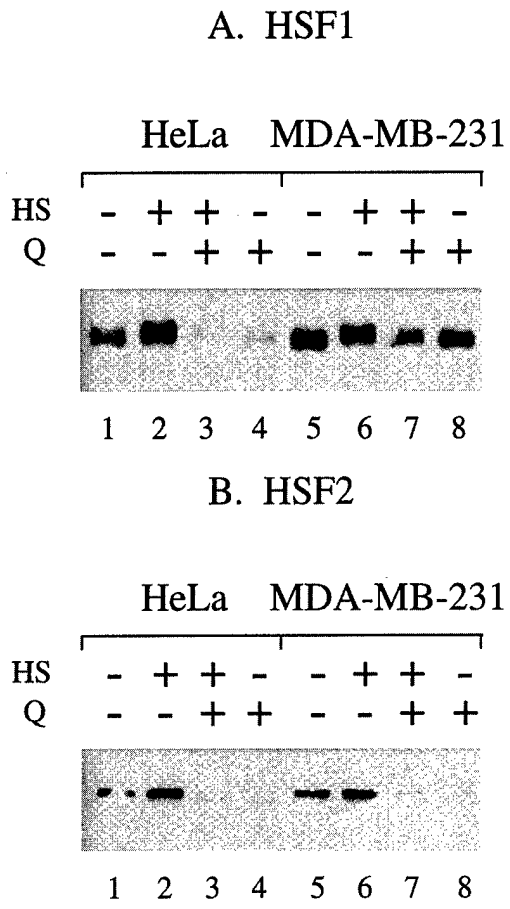

FIG. 5. HSF1 and HSF2 expression. Western blot analysis of HSF1 (Panel A) and HSF2 (Panel B) expression in HeLa and MDA-MB-231 cells. Protein extracts from untreated cells (lanes 1, 2 and lanes 5, 6), or cells treated with quercetin (lanes 3, 4 and lanes 7, 8) were subjected to Western blot analysis using antibodies to HSF1 and HSF2. In Panels A and B, the first four lanes correspond to HeLa cell extracts, while the last four lanes are extracts from MDA-MB-231 cells. HS indicates heat shock at 42°C for 1 hour, and Q denotes treatment with 100 μM quercetin.

HeLa cells were treated with 100 μM quercetin for 6 hours prior to heat shock at 42°C (26, 30), as in our study. Therefore, while it could be argued that quercetin's effect depends on the type and conditions of stress, our results suggest that this drug may have cell-type specificity. In some cells, it blocks HSF DNA-binding activity, while in other cells, it acts via other mechanisms to regulate hsp expression.

Candidate sites of regulation by quercetin in breast cancer cells include the activation of HSF, and/or the initiation of HSP transcription. Activation of eukaryotic HSFs involves multiple steps involving trimerization, DNA-binding, and activation of transcriptional competence. HSF exists as an inactive monomer, which is rapidly converted to a trimer, capable of binding DNA, upon heat shock (22). While quercetin does not appear to affect the trimerization process, it inhibits DNA-binding in COLO 320DM and HeLa cells (26, 30). However, binding of HSF to HSEs is necessary but not sufficient for transcription of hsps (33). Quercetin may block additional modifications necessary for activation of HSF transcriptional competence. These modifications may include post-translational modifications, release of a regulatory molecule, conformational changes, interactions with other DNA-binding proteins, and/or interactions with small ligands or metabolites.

Post-translational modification via phosphorylation plays an important role in the transcriptional activation of HSF1 (22, 33, 34). Quercetin treatment decreases the phosphorylated form of HSF1 in HeLa (26) and erythroleukemia (31) cells. Interestingly, recent reports indicate that there are at least two types of phosphorylated HSF1, and although both can bind DNA, only one is capable of inducing gene transcription. Transcriptionally inert HSF1 is constitutively, but not inducibly serine-phosphorylated, while active HSF1 is both constitutively and inducibly serine-phosphorylated (35, 36). Thus it is conceivable that quercetin may block serine-phosphorylation, resulting in inactive HSF1 in breast cancer cells. However, there are other possible ways quercetin could interfere with HSF activation. The hsps themselves have been suggested as candidate regulatory molecules for HSF activity, and may be quercetin targets, although it is generally accepted that increasing levels of hsps are needed to deactivate HSF (37-39). Yet another possiblity is that quercetin may affect conformational changes of HSF, which are required for activation (40). Or quercetin could inhibit interactions of HSF with other DNA-binding proteins at the promoter of hsp genes, which may be necessary to facilitate both formation of the HSF/HSE complex and transcriptional activation (41, 42). Finally, quercetin may affect interactions of HSF with as yet unidentified small ligands or metabolites (43). Clearly, these steps in HSF activation need to be investigated further.

In summary, our results demonstrate that quercetin inhibits the heat-induced expression of hsps, but not HSF DNA-binding or HSF1 expression in human breast cancer cells. While HSF2 expression was inhibited by quercetin treatment, it is probably not responsible for the decrease in hsp expression, as it is mainly associated with developmental regulation of hsp expression (44). Instead, we speculate that the mechanism by which quercetin inhibits hsp expression in breast cancer cells involves regulation of another step in HSF1 activation, since it does not alter DNA-binding activity. Furthermore, this study suggests that quercetin acts in a cell-type specific manner using HeLa and MDA-MB-231 under the same experimental conditions. An understanding of this mechanism may aid in the screening and design of future, specific therapeutic agents that could be used to inhibit hsps for the treatment of drug-resistant breast tumors.

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Hsp27 Overexpression Inhibits Doxorubicin-Induced Apoptosis in Human Breast Cancer Cells

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Running Title: Hsp27 inhibits apoptosis

Key words: hsp27, breast cancer, doxorubicin, apoptosis, topoisomerase II

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Submitted to Breast Cancer Research and Treatment

Summary

Previously we demonstrated that heat shock protein 27 (hsp27) overexpression confers resistance to the chemotherapeutic agent doxorubicin in MDA-MB-231 breast cancer cells. Since induction of apoptosis is one underlying mechanism of chemotherapeutic drug action, we investigated the effect of hsp27 overexpression on doxorubicin-induced apoptosis, finding that hsp27 protects MDA-MB-231 cells from apoptosis. We also examined expression of the doxorubicin target, topoisomerase II (topo II), in control and hsp27-overexpressing stable transfectants, as topo II expression is important for both drug sensitivity and the initiation of apoptosis by doxorubicin. The relative levels of both topo IIα and β were higher in the controls than the hsp27-overexpressing clones, suggesting that the apoptotic protective effect of hsp27 overexpression in MDA-MB-231 cells is associated with altered topo II expression.

Introduction

Breast cancers develop resistance to many chemotherapeutic drugs including the anthracycline doxorubicin, and the development of resistance is a major obstacle to the successful treatment of this disease (1). One mechanism of drug resistance occurs at the primary drug target interaction, and can involve decreased drug uptake, increased drug efflux, or an altered level of the intracellular drug target (2). For example, a decrease in the cellular levels of topo II hinders the efficacy of topo II inhibitors like doxorubicin (3). Another mechanism of drug resistance occurs downstream of the drug target interaction and involves suppression of apoptosis (1). Two examples of drug resistance via suppression of the apoptotic pathway involve expression of mutated p53 (4), and bcl-2 (5). In both cases, drug treatment elicited the same levels of cellular damage, eg. DNA strand breaks, as in cells lacking these two genes, but apoptosis was found to be suppressed.

Expression of the small heat shock protein 27, hsp27, is associated with the response of cancer cells to heat shock and chemotherapeutic drugs. For example, induction of hsp expression in MCF-7 and MDA-MB-231 breast cancer cells following heat shock treatment resulted in specific resistance to doxorubicin, but not colchicine, cisplatin, 5-fluorouracil, actinomycin D, or methotrexate (6), and selective overexpression of hsp27 in MDA-MB-231 cells also conferred 3-fold resistance to doxorubicin (7). Similarly, Chinese hamster ovary cells overexpressing hsp27 were resistant to doxorubicin, but not 5-fluorouracil, although hsp27 also protected these cells from colchicine and vincristine (8). Hence, hsp27 protects cancer cells against doxorubicin and other chemotherapeutic drugs, although the mechanism of action is not yet understood.

We know that doxorubicin inhibits DNA topoisomerases, enzymes that control DNA topology by cleaving and rejoining DNA strands and passing other DNA strands through the transient gaps (3). The inhibition of topoisomerase activity by doxorubicin, stabilizes the topoisomerase-DNA intermediate complex, and ultimately leads to cellular apoptosis. Here we investigated the effect of hsp27 overexpression on doxorubicin-induced apoptosis. We show that hsp27 inhibits apoptosis in MDA-MB-231 cells, an effect that is not associated with changes in

expression of the apoptosis-associated bcl-2 family of proteins. Since doxorubicin targets topo II, we also examined topo II expression in MDA-MB-231 cells. Our results demonstrate decreased topo II expression concomitant with hsp27 overexpression, providing one potential mechanism to explain the specific resistance of MDA-MB-231 breast cancer cells to doxorubicin. Furthermore, since targeting of topo II is an important step in the initiation of apoptosis (9), decreased topo II expression may protect breast cancer cells against doxorubicin-induced apoptosis.

Materials and Methods

Cells and cell culture

The MDA-MB-231 human breast cancer clones used in this study, vector control transfectants C1 and C2, and stable hsp27-overexpressing clones 19 and 12(2), were generated as previously described (10), and maintained in MEM supplemented with 10% fetal bovine serum, 6 ng/ml insulin, and 25 μg/ml gentamicin sulfate. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and periodically tested for Mycoplasma contamination (Bonique Laboratories, Saranac Lake, NY).

DNA fragmentation assay

Cells were plated at a density of 1.25 x 10^6 cells per T-75 flask. After 48 hours of growth, cells were treated with 0, 0.05, or 0.1 μg/ml doxorubicin for 48 hours, and a DNA fragmentation assay was performed as described (4). Briefly, approximately 5 x 10^6 cells were harvested using trypsin, washed with cold PBS, resuspended in cold buffer 1 (0.15M NaCl, 10mM Tris (pH7.4), 2mM $MgCl_2$, 1mM DTT, 0.5% NP-40), and incubated on ice for 40 minutes. Following centrifugation at 1200 rpm for 5 minutes at 4°C, the pellet was resuspended in cold buffer 2 (0.35M NaCl, 10mM Tris (pH 7.4), 2mM $MgCl_2$, 1mM DTT), incubated on ice for 20 minutes, and again centrifuged. DNA in the supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol, and precipitated overnight at -20°C with 2.5 volumes of 100% ethanol and 0.01M $MgCl_2$. The precipitates were centrifuged for 40 minutes and washed once with

70% ethanol. The air-dried DNA pellet was resuspended in 20 µl Tris-EDTA buffer (pH 8.0) containing 50 µg/ml DNase-free RNase A, and incubated at 37°C for 2 hours, followed by the addition of 1 mg/ml proteinase K and incubation at 37°C for 30 minutes. Fragmented DNA was visualized on 1.5% agarose gels in the presence of 0.5 µg/ml ethidium bromide.

ELISA apoptotic assay

Cells were plated at a density of 2.5 x 10^5 cells/well in a 6-well plate. After overnight growth, cells were treated with 0, 0.05, or 0.1 µg/ml doxorubicin for 48 hours, and apoptosis determined with the Cell Death Detection ELISA Assay (Boehringer Mannheim, Indianapolis, IN). Briefly, cells from one well of a 6-well plate were harvested using trypsin, washed with MEM, then the assay performed as per the manufacturer's instructions.

Western blot analysis

For immunoblot analysis of hsp levels in the transfectants, total cellular extracts were prepared using solubilization in 5% SDS. Extracts for the analysis of bcl-2 family members were prepared in 0.15M NaCl, 10mM Tris (pH 7.4), 5mM EDTA, 1% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 0.23 units/ml aprotinin, and 10 µM leupeptin, and cellular extracts for topo IIα and β were prepared using a modified RIPA lysis buffer containing 0.15M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris (pH 7.6), 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.1 mg/ml sodium orthovanadate, and 2 µg/ml aprotinin. In all cases equal amounts of protein (50 µg for hsp27, and 100 µg for bcl-2 members and topo IIα or β) were subjected to electrophoresis in 10% (for hsp27), 12.5% (for bcl-2 family members), or 6% (for topo IIα and β) polyacrylamide gels, and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Equal protein loading was ascertained by Ponceau S (Sigma, St. Louis, MO) staining of blotted membranes. Immunostains were developed using the Enhanced Chemiluminescence (ECL) system (DuPont, Boston, MA). Primary antibodies used in this study were anti-hsp27 (Neomarkers, Freemont, CA); anti-bcl-2, bak, bclx, mcl-1, bag, and bax, which

were kind gifts from Dr. J. C. Reed (Salk Institute, La Jolla, CA); anti-topo IIα (TopoGEN, Inc., Columbus, OH); and anti-topo IIα/β (Oncogene Research Products, Cambridge, MA). Fold protein expression differences between control transfectants C1 and C2, and stable hsp27-overexpressing clones 19 and 12(2), were determined by densitometric scanning of immunoblots from three independent experiments.

Cell cycle analysis

Cells were plated at a density of 1.25 x 10^6 cells per T-75 flask. After 48 hours of growth, cells were harvested for cell cycle analysis. In brief, 1 x 10^6 cells were harvested, washed with PBS, and stained with a modified Krishan reagent (11) (50 μg/ml propidium iodide in a hypotonic sodium citrate solution with 0.3% NP-40 and 1.0 mg/ml RNase A). All samples were analyzed with an EPICS ELITE flow cytometer (Coulter Cytometry, Miami, FL).

Results

Inhibition of doxorubicin-induced apoptosis

MDA-MB-231 breast cancer cells which express low levels of endogenous hsp27 were transfected with a plasmid coding for human hsp27 cDNA in the sense orientation, and the clones were screened for hsp27 overexpression by Western blot analysis (10). The range of hsp27 overexpression varied from 3- to 6-fold [clones 19, and 12(2)] (Figure 1A), as evaluated by densitometric scanning of the immunoblots as compared to control-transfected cells (clones 1 and 2).

There is increasing evidence that many chemotherapeutic drugs are effective primarily because they activate apoptosis (1). We previously demonstrated that hsp27 overexpression confers 3-fold resistance to doxorubicin (7) in breast cancer cells, but the mechanism by which hsp27 protected cells was unknown. In order to determine whether hsp27's protective effect enables breast cancer cells to circumvent doxorubicin-associated apoptosis, we investigated DNA fragmentation and morphological changes in hsp27-overexpressing MDA-MB-231 cells.

Treatment of control cells (C1 and C2) for 48 hours with either 0.05 or 0.1 μg/ml doxorubicin resulted in extensive DNA fragmentation, evident as a ladder of fragments with multiples of 200 base pair subunits, a characteristic feature of apoptotic cell death, while hsp27-overexpressing transfectants [19 and 12(2)] did not exhibit this cleavage pattern (Figure 1B). As additional evidence of hsp27's effect on apoptosis, we also performed the fragmentation assay using cells treated with two other drugs, etoposide and cycloheximide, and found that hsp27 overexpression also inhibited apoptosis induced by these agents (data not shown). At the morphological level, nuclear condensation, cell shrinkage, and membrane blebbing typical of apoptosis were also observed with drug treatment of the control cells; in contrast, hsp27-overexpressing transfectants maintained a normal morphology (data not shown).

In addition to the fragmentation assay, we conducted a cell death detection ELISA, which measures cytoplasmic histone-associated DNA fragments after drug-induced apoptosis. The amount of DNA fragments generated within individual clones is expressed as an Enrichment Factor (Table 1), derived by dividing the absorbance of cells treated with doxorubicin by the absorbance of cells treated with no doxorubicin, and used to compare the controls to hsp27-overexpressing transfectants. Hsp27-overexpressing cells demonstrated 3- to 5-fold lower levels of histone-associated fragments than control cells, following exposure to 0.05 or 0.1 μg/ml doxorubicin for 48 hours (Table 1). Hsp27 overexpression clearly inhibits doxorubicin-induced apoptosis, although it does not completely block this process, as is evident by the increase in the amount of histone-associated fragments at the higher drug dose (Table 1, 0.1 μg/ml DOX). Thus, hsp27 overexpression results in a decrease or delay in activation of the apoptotic cell death program.

Expression of bcl-2 apoptotic pathway family members

Since members of the bcl-2 multigene family play important roles in regulating the apoptotic process (12), we next investigated the expression of individual family members in MDA-MB-231 cells. Western blot analysis revealed that levels of the pro-apoptotic proteins, bak, or bax (Figure 2), $bclx_S$ (data not shown) and the anti-apoptotic proteins, mcl-1, or bag (Figure 2), $bclx_L$ (data not

shown) were the same between control and hsp27-overexpressing transfectants. In contrast, the level of bcl-2 was reduced approximately 2-fold in the hsp27-overexpressing clones (Figure 2), as determined by densitometric scanning of the immunoblots. Since bcl-2 is mainly anti-apoptotic in function (12), its reduction is not consistent with our observation that hsp27 protects MDA-MB-231 cells from apoptosis, suggesting that bcl-2 and the bcl-2 apoptotic pathway, may not be directly involved in doxorubicin resistance in our cells.

Expression of topo IIα and topo IIβ

There are two isoforms of topo II, topo IIα and topo IIβ, and both are targeted by doxorubicin (3). Resistance to doxorubicin can result from decreased levels of topo II expression (3), and the targeting of topo II by doxorubicin is an important step in the induction of doxorubicin-associated apoptosis (9). Therefore, we analyzed relative expression levels of topo IIα and topo IIβ in control and hsp27-overexpressing MDA-MB-231 cells (Figure 3). Protein expression of topo IIα and β was reduced approximately 1.5- to 2-fold in the hsp27-overexpressing transfectants, as compared to control cells without drug (Figure 3A), determined by densitometric scanning of the immunoblots. In addition, there was an overall increase in the amount of topo IIα and topo IIβ expression with drug treatment in all the cells (compare Figure 3 panel A with panel B). After treatment for 48 hours with 0.1 μg/ml doxorubicin, the decrease in topo IIα and topo IIβ expression was even more apparent than in untreated cells, with an approximate 2-fold reduction in their levels in the hsp27-overexpressing transfectants (Figure 3B), evaluated by densitometric scanning. Thus hsp27 overexpression represses topo II levels contributing to the observed differences in drug sensitivity.

Effect of hsp27 overexpression on cell cycle phase distribution

We previously demonstrated that hsp27 overexpression results in increased anchorage-dependent and anchorage-independent growth (7). We next examined whether increased proliferation was due to changes in the cell cycle phase distribution. Flow cytometric analysis

demonstrated that there was no difference in the population of control or hsp27-overexpressing cells in any phase of the cell cycle (Table 2).

Discussion

In this paper, we show that hsp27 overexpression protects breast cancer cells from doxorubicin-induced apoptosis. Transfection of a human hsp27 cDNA into MDA-MB-231 cells resulted in a marked reduction in apoptotic cell death after doxorubicin treatment. These results provide evidence of a strong positive association between hsp27 expression and resistance to apoptosis in breast cancer cells, consistent with results in murine cells (13, 14). Using murine fibrosarcoma cells, Mehlen et al. (13) demonstrated that overexpression of hsp27 blocks Fas/APO-1-and staurosporine-mediated cell death. Using murine embryonic stem cells, Mehlen et al. (14) also showed that inhibition of hsp27 expression drives differentiating cells toward cell death because of increased apoptosis. Therefore, hsp27 can repress specific cell death genes that are necessary for apoptosis and which are induced by different agents.

Previously, we and others have shown that hsp27 overexpression protects cells from doxorubicin treatment (7, 8), and that hsp27 overexpression is a bad prognostic marker in a subset of breast cancer patients (15), but limited information concerning the physiological function of hsp27 made it difficult to ascertain its mechanism of activity. Here we show that overexpression of hsp27 in MDA-MB-231 cells is associated with decreased topo IIα and β protein expression. Consistent with these results, at the RNA level, we found decreased topo II expression in untreated hsp27 transfectants as compared to controls and overall increased topo II expression with doxorubicin treatment in both controls and transfectants using the Clontech Atlas cDNA Expression Array (data not shown) (16). These results are clinically important because decreased topo II expression has been shown to be correlated with resistance to doxorubicin, both in cell lines and tumors, presumably due to decreased substrate on which the drug can act (17, 18). In human breast tumors, topo IIβ is expressed in a greater proportion of tumor cells than topo IIα (19), suggesting that topo IIβ might be a more significant therapeutic target for topo II-interfering drugs

than topo IIα. In contrast, a small study of tumors from breast cancer patients (n = 15) demonstrated that the majority of doxorubicin-sensitive tumors expressed higher levels of topo IIα, with all of the doxorubicin-resistant tumors expressing undetectable or low levels of topo IIα (18). Thus both topo IIα and β levels may be important determinants of doxorubicin-sensitivity.

In addition to conferring resistance to doxorubicin, hsp27 and topo IIα expression, but not topo IIβ, are also important positive factors for cellular proliferation. Hsp27 overexpression increases cell growth (7, 20), and it is known that rapidly proliferating tumor cells have elevated levels of topo IIα (19). Furthermore, topo IIα levels are positively correlated with the percentage of tumor cells in S-phase in patients (19). The association between hsp27 expression and growth, however, appears uncoupled from the protective effect of hsp27 to doxorubicin, as increased proliferation would be expected to result in increased drug sensitivity, not increased resistance, as we found in our transfectants. Furthermore, there does not appear to be a positive relationship between topo IIα, hsp27, proliferation, and S-phase in MDA-MB-231 cells, since we found that overexpression of hsp27 was correlated with a decrease in topo IIα and β expression, and no difference in percent of cells in S-phase. It is tempting to speculate that hsp27 is associated with a poor prognosis in patients because it protects breast cancer cells from doxorubicin-induced apoptosis by decreasing topo II expression, in addition to influencing tumor cell growth.

It has also been reported that treatment of HL-60 cells with bufalin leads to a decrease in topo II mRNA and protein levels (21). In contrast, cisplatin treatment induces topo II expression in colon carcinoma cells, as well as increasing hsp27 levels, presumably due to modulation of the cell cycle (20). However, cisplatin treatment in these cells leads to an increase in sensitivity to the drug, suggesting that the increase in topo II target dominated over the protective effect of hsp27 overexpression. Thus in agreement with our results, topo II and hsp27 expression can have opposite effects on the drug sensitivity of cells.

Clearly hsp27 is an important determinant of doxorubicin resistance, and possibly resistance to other chemotherapeutic drugs (7, 8). While decreased topo II expression contributed to the ability of hsp27-overexpressing breast cancer cells to resist drugs which target topo II, the

capacity of hsp27 to block apoptosis induced by drugs such as staurosporine (13) that act through other cell death pathways suggests that hsp27 may exert additional intervention at the level of an early or general mediator of cell death, perhaps by modulation of regulatory genes like p53 and bcl-2. But since MDA-MB-231 cells have a mutated p53 (22), this gene is an unlikely candidate for modulation by hsp27. Likewise, the bcl-2 family may not be involved in hsp27's protective effect since we were unable to show major differences in expression of these family members important for the apoptotic phenotype (eg. decreased expression of the pro-apoptotic bax protein or increased expression of the anti-apoptotic bcl-2 protein), concomitant with hsp27 overexpression. These results agree with observations by Guenal et al. (23) indicating that bcl-2 and hsp27 act at several levels to prevent apoptosis, and although there are similarities between their protective effects, their functions do not overlap. Recently; hsp27 was shown to protect murine fibrosarcoma cells from death stimulated through the TNFα (24) and the Fas/APO-1 pathways (13), suggesting that hsp27 regulates the expression or activity of a gene or genes downstream of these receptors. Breast cancer cells can be sensitive or resistant to TNFα, and this is associated with their estrogen receptor status (25), although the mechanisms controlling TNFα sensitivity in breast cancer cells are not understood. In contrast, breast cancer cells are uniformly resistant to Fas-mediated apoptosis, irrespective of their estrogen receptor status (26), and this is due to modulation of the Fas/APO-1 receptor expression rather than loss of downstream components of the pathway. Thus, hsp27 may not modulate TNFα or Fas/APO-1 receptor expression in breast cancer cells, but may instead regulate the activity of a gene or genes downstream of these receptors. Alternatively, hsp27 expression may exert a general protective role in the cell by reducing topo II levels as we found, or by decreasing intracellular reactive oxygen species (24), which has also been shown to inhibit apoptosis (27).

In conclusion, we provide evidence that hsp27 protects breast cancer cells from doxorubicin-induced apoptotic cell death. We would like to suggest that hsp27 overexpression results in a decreased ability to activate apoptosis and the cell death program, independent of p53 and bcl-2 in these cells. Hsp27 overexpression thus represses specific cell death genes, as yet

unknown, necessary for apoptosis along with reducing doxorubicin's target, topo IIα and ß. We envision that these studies will help facilitate the identification of novel targets downstream of hsp27's effect for the development of more specific chemotherapeutic agents designed to circumvent hsp27 and specifically launch the apoptotic machinery of the cell.

Acknowledgments

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Table 1. Fold Apoptosis Expressed as an Enrichment Factor[a,b]

	No DOX	0.05 µg/ml DOX	0.1 µg/ml DOX
C1	1.0	5.0	7.8
C2	1.0	4.5	7.8
19	1.0	1.0	2.0
12(2)	1.0	1.0	2.3

[a]The amount of DNA fragments generated within individual clones is expressed as an Enrichment Factor, derived by dividing the absorbance of cells treated with doxorubicin by the absorbance of untreated cells. Control (C1 and C2) and hsp27-transfected [19 and 12(2)] cells were either untreated (No DOX) or treated with 0.05 or 0.1 µg/ml doxorubicin for 48 hours prior to ELISA apoptotic assay.

[b]Data shown represent the mean for three independent experiments.

Table 2. Effect of hsp27 overexpression on cell cycle phase distribution[a,b]

	%G0/G1	%S Phase	%G2/M
C1	45.4	38.7	15.9
C2	48.7	37.3	14.0
19	44.4	36.7	18.9
12(2)	44.8	39.5	16.2
P value	ns	ns	ns

[a]Control (C1 and C2) and hsp27-transfected [19 and 12(2)] cells were analyzed for DNA content by flow cytometric analysis as described in 'Materials and Methods'.

[b]Data shown represent the mean for three independent experiments. The P value was greater than 0.1 in all cases.

Figure Legends

Figure 1. Inhibition of doxorubicin-induced apoptosis in hsp27-transfected MDA-MB-231 cells. Panel A, hsp27 expression in transfected MDA-MB-231 clones. Western blot analysis of hsp27 levels in control (C1, C2) and hsp27-transfected [19, 12(2)] cells was performed in 10% polyacrylamide gels as described in "Materials and Methods." The molecular weight of hsp27 is indicated on the right side of the immunoblot. Panel B, induction of genome digestion by doxorubicin. MDA-MB-231 transfectants were either untreated (No DOX) or treated with 0.05 or 0.1 μg/ml doxorubicin for 48 hours. Cellular DNA was extracted and analyzed by agarose gel electrophoresis. Data shown are representative of three independent experiments.

Figure 2. Expression of bcl-2 family member proteins. Western blot analysis of bak, bax, mcl-1, bag, and bcl-2 in control (C1, C2) and hsp27-transfected [19, 12(2)] cells was performed in 12.5% polyacrylamide gels as described in "Materials and Methods" using antibodies for various bcl-2 family members. The molecular weights are indicated on the right side of the immunoblots. Data shown are representative of two independent experiments.

Figure 3. Expression of topo IIα and β. Western blot analysis of topo IIα and β in control (C1 and C2) and hsp27-transfected [19 and 12(2)] cells was performed in 6% polyacrylamide gels as described in "Materials and Methods" using antibodies for topo IIα or β. Panel A, no doxorubicin treatment (No DOX). The topo IIβ antibody crossreacts with topo IIα, which was a visible band below topo IIβ on a darker exposure of the topo IIβ blot. Panel B, doxorubicin treatment. Cells were treated with 0.1 μg/ml doxorubicin for 48 hours prior to Western blot analysis. The topo IIα band is shown below topo IIβ. The molecular weights are indicated on the right side of the immunoblots.

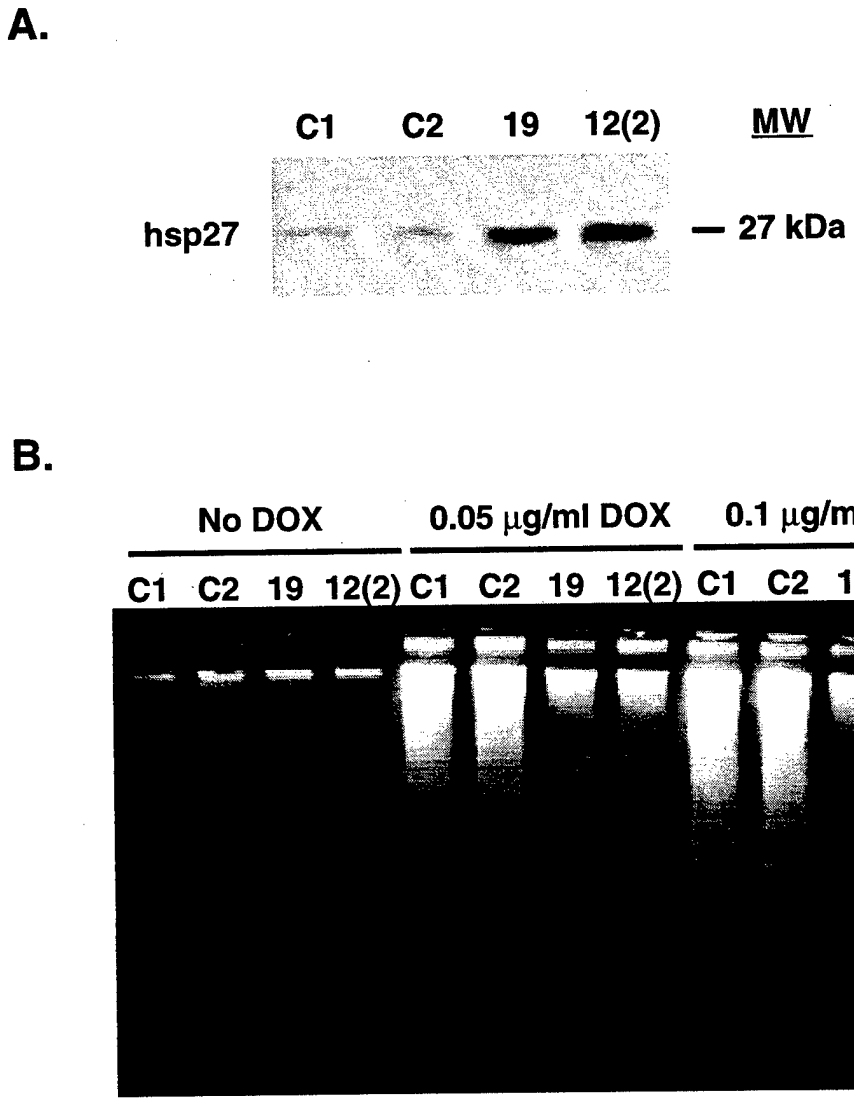

A.
C1
C2
19
12(2)
MW
hsp27
— 27 kDa
B.
No DOX
0.05 μg/ml DOX
0.1 μg/ml DOX
C1 C2 19 12(2) C1 C2 19 12(2) C1 C2 19 12(2)

Figure 1

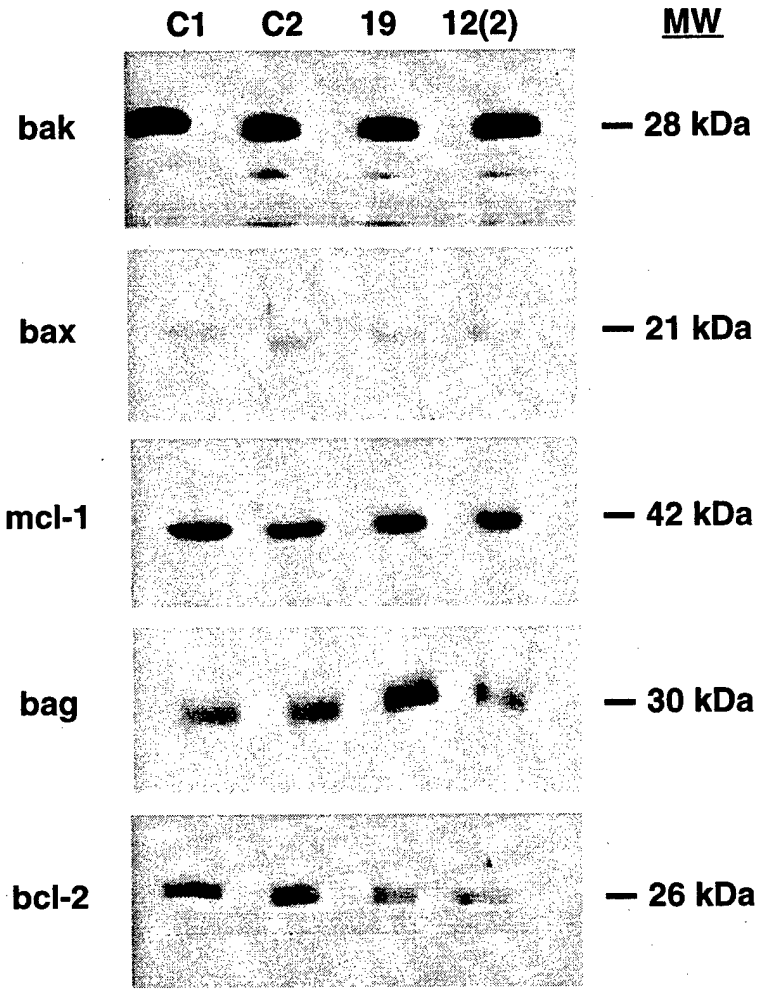

C1
C2
19
12(2)
MW
bak
— 28 kDa
bax
— 21 kDa
mcl-1
— 42 kDa
bag
— 30 kDa
bcl-2
— 26 kDa

Figure 2

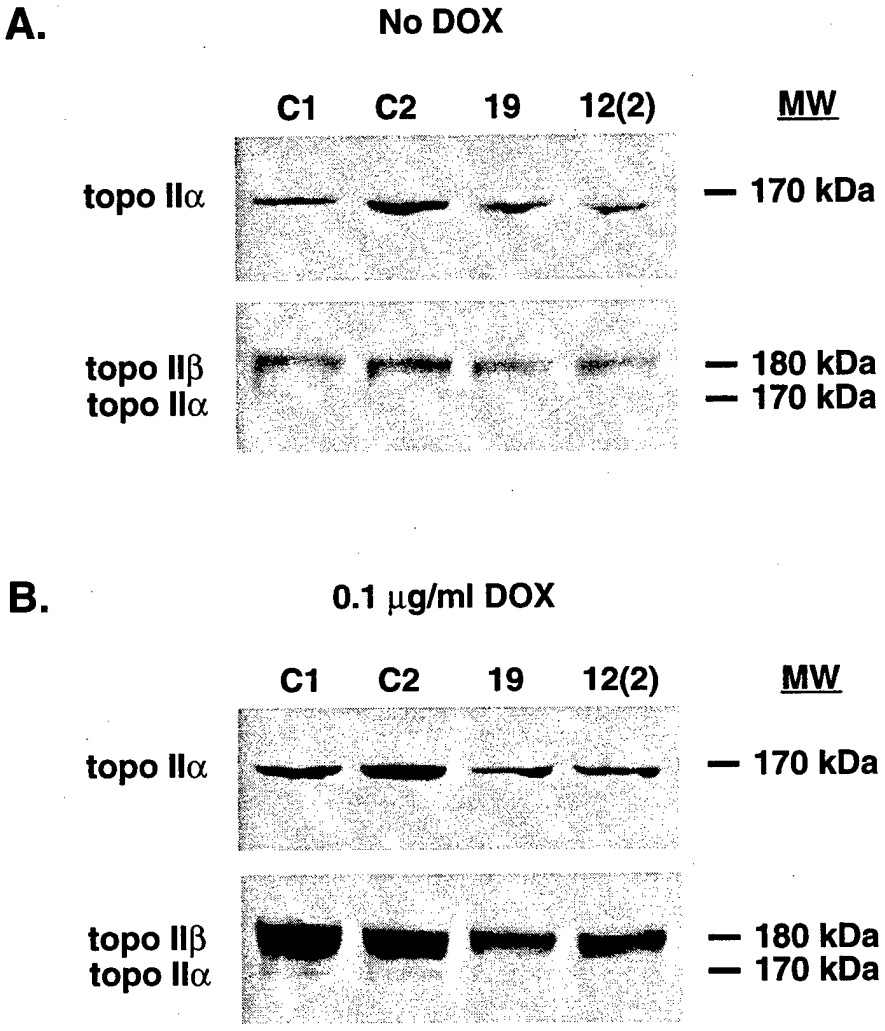
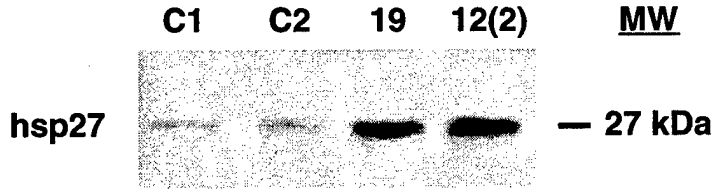
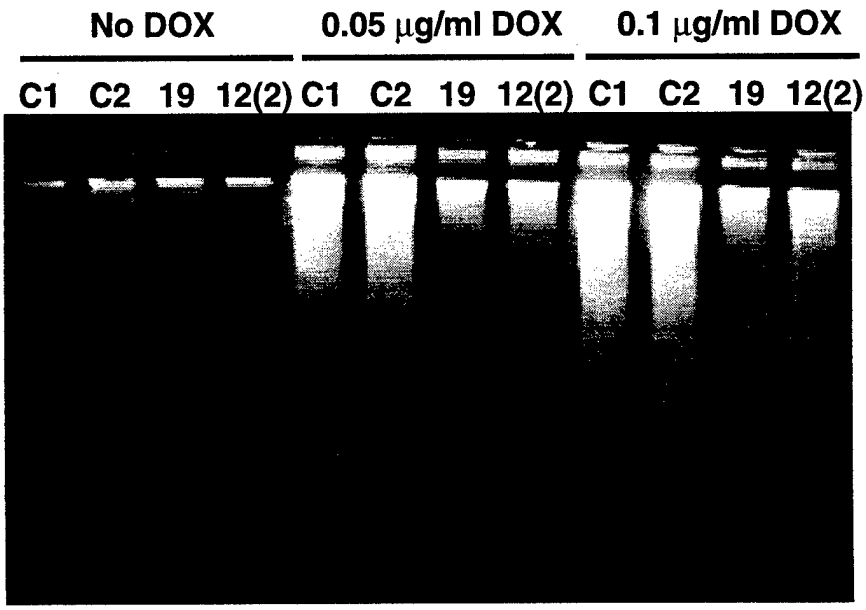

A.
No DOX
C1
C2
19
12(2)
MW
topo IIα
— 170 kDa
topo IIβ
topo IIα
— 180 kDa
— 170 kDa
B.
0.1 μg/ml DOX
C1
C2
19
12(2)
MW
topo IIα
— 170 kDa
topo IIβ
topo IIα
— 180 kDa
— 170 kDa

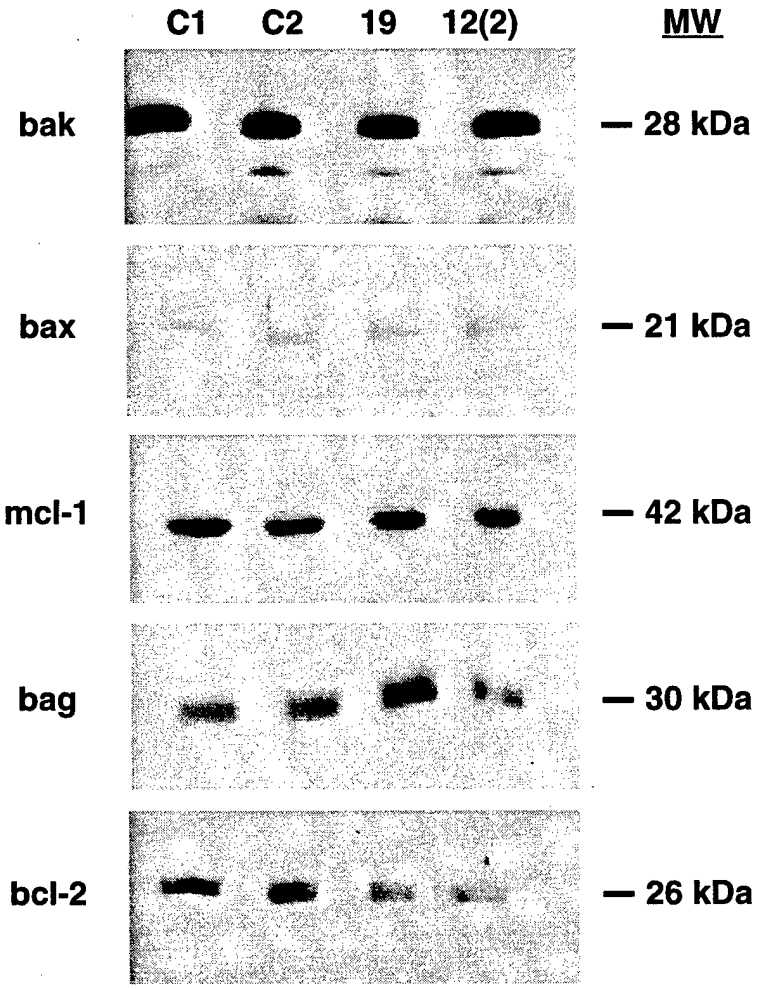
Figure 3

A.

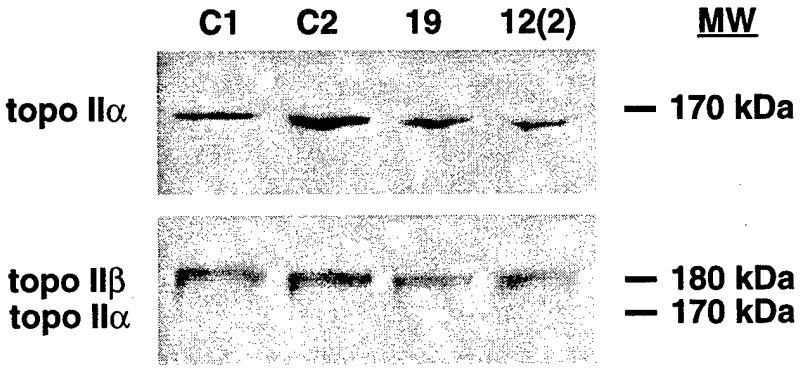

C1
C2
19
12(2)
MW
hsp27
— 27 kDa

B.


No DOX
0.05 μg/ml DOX
0.1 μg/ml DOX
C1 C2 19 12(2) C1 C2 19 12(2) C1 C2 19 12(2)


C1
C2
19
12(2)
MW
bak
— 28 kDa
bax
— 21 kDa
mcl-1
— 42 kDa
bag
— 30 kDa
bcl-2
— 26 kDa

A. No DOX



B. 0.1 μg/ml DOX